THE ROLE OF REACTIVE OXYGEN SPECIES IN THE RETROGRADE CHLOROPLAST-NUCLEUS SIGNALLING PATHWAY

A Thesis submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Biology

University of Saskatchewan

Saskatoon

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ABSTRACT

Photosynthetic organisms are particularly susceptible to photooxidative stress, because they are dependent on light energy for converting carbon dioxide into organic compounds and as a by-product, generate high levels of oxygen in the chloroplast. While photosynthetic eukaryotes exhibit altered nuclear gene expression in response to changes in the production of reactive oxygen species (ROS) in the chloroplast, little is known about how this signal is transmitted from the chloroplast to the nucleus. In the green alga *Chlamydomonas reinhardtii*, the cytosolic *GLUTATHIONE PEROXIDASE 5* gene (*GPX5*) is known to be up-regulated at the level of transcription in response to singlet oxygen. Previous studies have shown that when the promoter region of *GPX5* is fused to the *ARYLSULFATASE 2* gene (*ARS2*), an effective reporter system can be generated and used to study *GPX5* expression. This system was used in this study to generate a stably transformed *C. reinhardtii* strain which expresses the ARS2 protein in a singlet oxygen-dependent manner. Using the strain of *C. reinhardtii* harbouring a singlet oxygen-sensitive promoter gene, secondary mutagenic screen was performed. This allowed identification of mutant cell lines that were unable to up-regulate *GPX5-ARS2* fusion expression, based on ARS2 activity, in response to singlet oxygen production. In one of these lines, the mutation was subsequently localized to the first exon of the *PSBP-like* gene (*PSBP2*), and this line was designated as *psbP2*. The *PSBP2* gene is part of a small gene family in *C. reinhardtii* that is conserved in higher plant species. While each member of the PSBP protein family contains a similar domain to the PSBP1 protein, which is a member of the oxygen evolving complex of photosystem II (PSII), the PSBP2 protein does not appear to be involved in PSII function. While *psbP2* does not produce greater ARS2 activity in response to singlet oxygen, it still accumulates both the *GPX5-ARS2* and native *GPX5* transcripts when challenged by photosensitizer exposure, although at lower levels than the original lines. It was demonstrated that the PSBP2 protein is involved in transmitting information related to the accumulation of singlet oxygen in the chloroplast to control the singlet oxygen-dependent *GPX5* driven *ARS2* expression in the nucleus and/or ARS2 activity through a post-transcriptional process in the cytoplasm.

ACKNOWLEDGEMENTS

This dissertation crowns the work conducted in Dr. Ken Wilson and Dr. Gordon R. Gray labs. It would not be possible to accomplish without professional and often personal help and support I received from both of them. I am glad I could work not only for but most importantly with them.

I would also like to thank the advisory committee members, Dr. Peta Bonham-Smith, Dr. Jitao Zou, and Dr. Hong Wang for constructive criticism and invaluable input into the research process. Your knowledge and diverse approach helped me to see different aspects of my research and solve many difficult problems.

I am thankful for many interesting discussions and support from the faculty members: Jack Gray, Chris Todd, Jose Andres, Hugo Cota-Sanchez, Art Davis, Carlos Carvalho, Susan Kaminskyj, Yangdou Wei, and David C. Logan. I would also like to say thank you to Neil Chilton for an opportunity to work in his lab, it was very helpful and an interesting experience.

Working as a teaching assistant sometimes can prove to be quite challenging unless you have an instructor like Sheri Fisher on your side that you can learn from and work with.

Thank you to past and present members of the departmental staff for professional help and the ability to solve almost any problem: Bonita Wong, Deidre Wasyliw, Joan Virgl, Leona Leason, and Shirley Hleck. I appreciate all the work you have done on my behalf.

For all the technical support, thank you to Guocheng Liu, Marlynn Mierau, Gordon J. Holtslander, and Jeaniene Smith.

I would also like to thank two wonderful people from ISO: Jacquie McDermott and Derek Tannis, for all the support, dedication and extraordinary work they do on behalf of international students.

Lastly, I would like to thank Veronica Duran, for the support, understanding, encouragement, optimism, calmness, cheerfulness, and much more.

Ad augusta per angusta

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LIST OF ABBREVIATIONS

$O_2^{\bullet-}$	superoxide anion
$^1O_2$	singlet oxygen
APX	ascorbate peroxidase
ARS2	ARYLSULFATASE 2
bya	billion years ago
cDNA	complementary DNA
Chl	chlorophyll
ChlH	H subunit of magnesium chelatase
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
EtBr	ethidium bromide
ETC	electron transfer chain
FB(B)S	fast blue B salt (tetrazotized-o-dianisidine)
GL	growth-light (50 µmol photons m^{-2} s^{-1})
GPX5	GLUTATHIONE PEROXIDASE 5 (*C. reinhardtii*)
GR	glutathione reductase
GSH	glutathione
GUN	genome uncoupled *A. thaliana* mutant
H_2O_2	hydrogen peroxide
HL	high-light (920 µmol photons m^{-2} s^{-1})
LHCB	protein of LHCII
LHCII	light harvesting complexes of photosystem II
LOOH	lipid hydroperoxide
MAPK	mitogen-activated protein kinase
MgProto	Mg-protoporphyrin IX
Mg-ProtoME	magnesium protoporphyrin IX monomethyl ester
Mg-ProtoME$_2$	magnesium protoporphyrin IX dimethyl ester
ML	moderate-light (100 µmol photons m^{-2} s^{-1})
mRNA	messenger RNA
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NR	neutral red

$N\text{-}SO_4$	α-naphthyl sulfate potassium salt
$OH^{\bullet}$	hydroxyl radical
Pchlide	protochlorophyllide
PQ	plastoquinone
Proto	protoporphyrin IX
Protogen	protoporphyrinogen IX
PSI	photosystem I
PSII	photosystem II
qRT-PCT	quantitative reverse transcriptase PCR
RACE-PCR	rapid amplification of cDNA ends PCR
RB	rose bengal
RBCS	nuclear genes encoding small subunit of RuBisCO
RESDA-PCR	restriction-enzyme-site-directed PCR
ROS	reactive oxygen species
RR	regulatory region
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SET	Serial Endosymbiosis Theory
-SH	thiol group
SOD	superoxide dismutase
sqRT-PCR	semi-quantitative reverse transcriptase PCR
TAP	tris-acetate-phosphate
t-BOOH	*tert*-butyl hydroperoxide
TP	tris-phosphate
TRX	thioredoxin
WT	wild-type

CHAPTER 1.
LITERATURE REVIEW

1.1. Introduction

Endosymbiotic processes were the defining events in the evolution of eukaryotic organisms. As current chloroplasts and mitochondria evolved on this course, the development of an efficient communication system between the organelle and nucleus was required. Such a signalling system is one of the most crucial factors for any "symbiotic consortium" to function properly. "Information" exchange between chloroplasts, mitochondria, and nucleus takes place by means of anterograde ("forward", nucleus-to-organelle) and retrograde ("backward", organelle-to-nucleus) signalling pathways. This bi-directional communication is necessary for coordination of organelles' development, function, and adjustments to changing environmental conditions.

There is evidence that the chloroplast can exert an effect on nuclear gene expression (Beck 2001). Five retrograde signalling pathways involved in chloroplast-to-nucleus communication have been proposed: product(s) of plastid protein synthesis (Adamska 1995), intermediates in tetrapyrrole biosynthesis (Chekounova *et al.* 2001; Surpin *et al.* 2002), chloroplast-generated H_2O_2 (Fryer *et al.* 2003; Karpinski *et al.* 1999), redox state of the photosynthetic electron transport chain (ETC) (Escoubas *et al.* 1995), and chloroplast-generated $^1O_2$ (Krieger-Liszkay 2005; op den Camp *et al.* 2003) which is the subject of the current investigation.

Singlet oxygen ($^1O_2$) is produced through the interaction of oxygen with excited-state chlorophyll (Bjorn 2008; Cadenas 1989; Vass and Cser 2009) and it is thought to be the major ROS involved in photooxidative damage (Triantaphylides *et al.* 2008). However, there is also evidence that production of $^1O_2$ in the chloroplast induces stress responses that do not seem to result merely from physicochemical damage caused by ROS (op den Camp *et al.* 2003). These responses appear to be the effect of induction of several stress-response pathways with the expression of specific genes being regulated to protect the cell from photooxidative damage (Gadjev *et al.* 2006; Laloi *et al.* 2007;

Ledford *et al.* 2007), although mechanisms of this signal transduction has not yet been elucidated.

In the green alga *Chlamydomonas reinhardtii*, a specific suite of nuclear genes are known to be up-regulated in response to $^1O_2$ (Ledford *et al.* 2007). Based on gene expression studies, cytoplasmic *GLUTATHIONE PEROXIDASE 5* (*GPX5*) exhibits the largest increase in transcript abundance in response to $^1O_2$ exposure, and this increase occurs specifically in response to $^1O_2$ (Fischer *et al.* 2009; Ledford *et al.* 2007; Leisinger *et al.* 2001).

Transcription of the *GPX5* gene is up-regulated in response to both endogenous $^1O_2$ and exogenously applied photosensitizers (Fischer *et al.* 2009; Ledford *et al.* 2007; Leisinger *et al.* 2001). However, to date no signalling mechanism has been identified that is responsible for the increase in *GPX5* mRNA accumulation above the basal level of expression occurring under low-light conditions (Leisinger *et al.* 2001).

The *GPX5* regulatory region (RR) fused with promoterless *ARYLSULFATASE 2* (*ARS2*) is an effective reporter system for monitoring ROS-mediated signalling pathway in *C. reinhardtii* (Leisinger *et al.* 2001). In the research presented here, the *GPX5-ARS2* reporter gene was used in a stably transformed strain of *C. reinhardtii*. A screen of *C. reinhardtii* insertional mutants, generated by secondary mutagenesis, identified a nuclear-localized *PSBP2* gene encoding a protein required for the $^1O_2$-dependent chloroplast-nucleus retrograde signalling.

1.2. Endosymbiosis and the evolution of bilateral chloroplast-nucleus communications

Life came into being on Earth in a non-oxygenated atmosphere (Canfield 2005; Kasting 1993) and although the timing of the origin of photosynthesis is still under dispute, it is generally accepted that it evolved in cyanobacteria 2.7 to 3.7 bya (billion years ago; Brasier *et al.* 2002; Brocks *et al.* 1999; Canfield 2005; Rosing and Frei 2004). There is no other known biological or non-biological process capable of producing molecular oxygen in quantities large enough to significantly change the Earth's atmospheric content (Blankenship and Hartman 1998). Thus, the rise in oxygen in the early atmosphere had to be a side effect of photosynthesis and today oxygen constitutes

21% of the atmosphere. It is estimated that about 10^{27} cells of cyanobacteria float on the surface water of the ocean (as phytoplankton), of which *Prochlorococcus* is considered to be not only the most numerous photosynthetic organism but also the most plentiful (on Earth in general) (Catling and Claire 2005; Partensky *et al.* 1999). While ancestral cyanobacteria were probably as abundant, their effect on the oxygen concentration in the atmosphere was delayed because of complicated chemistry and the chemically reducing character of the early Earth (Catling and Claire 2005). Thus, several hundred million years are thought to have passed between the proposed origin of oxygenic photosynthesis and the occurrence of detectable atmospheric O_2 (Catling and Claire 2005). Free molecular oxygen, because of its toxicity, doubtlessly caused one of the first great ecological shifts in the history of our planet. According to the geochemical data there were two major increases of O_2 during the history of the Earth. The first one occurred 2.4-2.3 bya during the Paleoproterozoic era when the atmospheric partial pressure of oxygen reached $>10^{-5}$ times that of the present atmospheric level (PAL; Bekker *et al.* 2004). The second increase took place around 1.0-0.6 bya during the Neoproterozoic to approximately 10-20% PAL (Canfield 1998). These two time periods are thought to reflect significant changes in Earth's climate, geochemistry, and chemical events, leading to the evolution of plants and animals (Canfield 1998; Catling and Claire 2005; Raymond and Segre 2006). The accumulation of oxygen in the atmosphere was a strong selective pressure, leading to the development of specific defence mechanisms (Corsetti *et al.* 2006; Knoll 2003; Kopp *et al.* 2005). Thus, some organisms survived and thrived in the oxygenated atmosphere because they developed means to manage and even take advantage of this potentially toxic gas.

To function, every organism has to maintain homeostasis, keeping its internal environment constant both metabolically within a cell and physiologically in terms of the whole organism. If external conditions are changing over time, for example the increasing concentration of molecular oxygen in the atmosphere, organisms have to adapt to survive by developing new characteristics. The development of endosymbiotic cooperation is thought to have originated during the period when oxygen was accumulating in the atmosphere (Falcon *et al.* 2010). From an evolutionary point of view, the development of an endosymbiotic system can lead to a faster way of developing new

characteristics than through the accumulation of mutations alone. The endosymbiotic partnership would allow the obligate anaerobic bacteria to inhabit oxygenic areas, because the aerobic partner kept cellular O_2 levels low due to respiratory processes (Margulis 1998). In a relatively quick and radically changing environment, as must have occurred during the rise of atmospheric oxygen, the acquisition of an endosymbiont which is capable not only of coping with this toxic gas but even of using it for metabolic processes, would have provided a much better chance of survival under this strong selective pressure. Thus, the precursors of mitochondria may have turned out to be extremely good at utilizing oxygen and as a result protecting the host cell from the otherwise toxic environment. Whether such an engulfment of aerobic bacteria happened once or many times is still under debate. However, most scientists agree that mitochondria (as well as chloroplasts) constitute a monophyletic group which means that all organisms containing them have a common ancestral lineage (Emelyanov 2001; Falcon *et al.* 2010). Thus, it seems that engulfing an aerobic bacteria that became the mitochondria was so beneficial that this lineage eliminated almost all other eukaryotes (deDuve 1996).

The origin of *Eukaryota* (organisms with nucleate organization, (Whittaker and Margulis 1978) can be explained by the Serial Endosymbiotic Theory (SET). This theory was first proposed by Mereschkowsky (1905, origin of chloroplasts) and Wallin (1927, origin of mitochondria). Although welcomed with great scepticism and ignored, it was revived by Sagan (1967) and Margulis (1993), and today is widely accepted. The SET (Figure 1.1) suggests that the transformations leading to the formation of chloroplasts and mitochondria were serial in nature, meaning that events did not take place at the same time (Margulis 1970, 1998). Mitochondria evolved from aerobic bacteria, which were absorbed by a host cell. Instead of being digested, the bacterial cell was kept as an endosymbiont and developed into the modern mitochondrion. It was very profitable for the host cell for the sake of removing damaging O_2 and producing ATP. The endosymbiont in turn was protected by the host and had stable life conditions (Margulis 1998). In the second step of the SET a mitochondria-containing cell absorbed an autotrophic prokaryotic cell (cyanobacteria). In this case the new occupant also became an endosymbiont and in time a chloroplast (Margulis 1970, 1998) as outlined in Figure

1.1. Margulis states that such symbiogenesis or symbiotic fusions were very common in the history of life evolution on Earth (Margulis 1993). Margulis also states that bacteria were the ancestors of all life on our planet, and in fact, they led to the creation of all living organisms known today (Margulis 1970, 1998). The cyanobacterial ancestry of chloroplasts has been studied using phylogenetic analysis. Based on the comparison of the cyanobacterial and chloroplast 16S rDNA and *rbcL* genes it was determined that chloroplasts constitute a monophyletic group and are most closely related to unicellular N_2-fixing cyanobacteria from the order of Chroococcales (Falcon *et al.* 2010).

After the primary endosymbiotic events, mitochondria and chloroplasts retained DNA encoding only a small number of proteins and depend on the vast majority of their proteome encoded in the nuclear genome. Therefore, they are regarded as semi-autonomous organelles, although in some organisms they have degraded to near non-functional relics (Martin 2005). Thus, most of the endosymbiont genes were transferred into the nucleus of their host, between the organelle, or were lost. This probably occurred simultaneously with the evolution of host regulatory systems. Possible reasons for the gene transfer into the nucleus are the more efficient DNA repair and decreased DNA damage due to metabolism in chloroplasts and mitochondria (Allen and Raven 1996; Martin and Herrmann 1998). Metabolism in organelles is characterized by highly reactive redox reactions and the production of reactive oxygen species (ROS) which can cause DNA mutations (Allen and Raven 1996). However, the question of why some genes remained in organelle genomes is unclear. It was proposed that some organelle proteins have to be produced in organelles because they cannot be imported due to their hydrophobic nature (von Heijne 1986). However, this does not seem to be the case. Precursors of the light harvesting protein of chlorophyll *b* are nuclear-encoded, hydrophobic polypeptides, yet they can be transported across the plastid envelope (Mullet 1988). Similarly, it was demonstrated that the gene encoding the large subunit of RuBisCO (ribulose-1,5-bisphosphate carboxylase /oxygenase) can be removed from the chloroplast, placed in the nucleus where following transcription and translation it is imported into the aqueous, stromal phase of the plastid where it is fully functional (Kanevski and Maliga 1994). Another study also showed that the large subunit of RuBisCO, as well as β subunit of chloroplast ATP synthase, could be imported into the

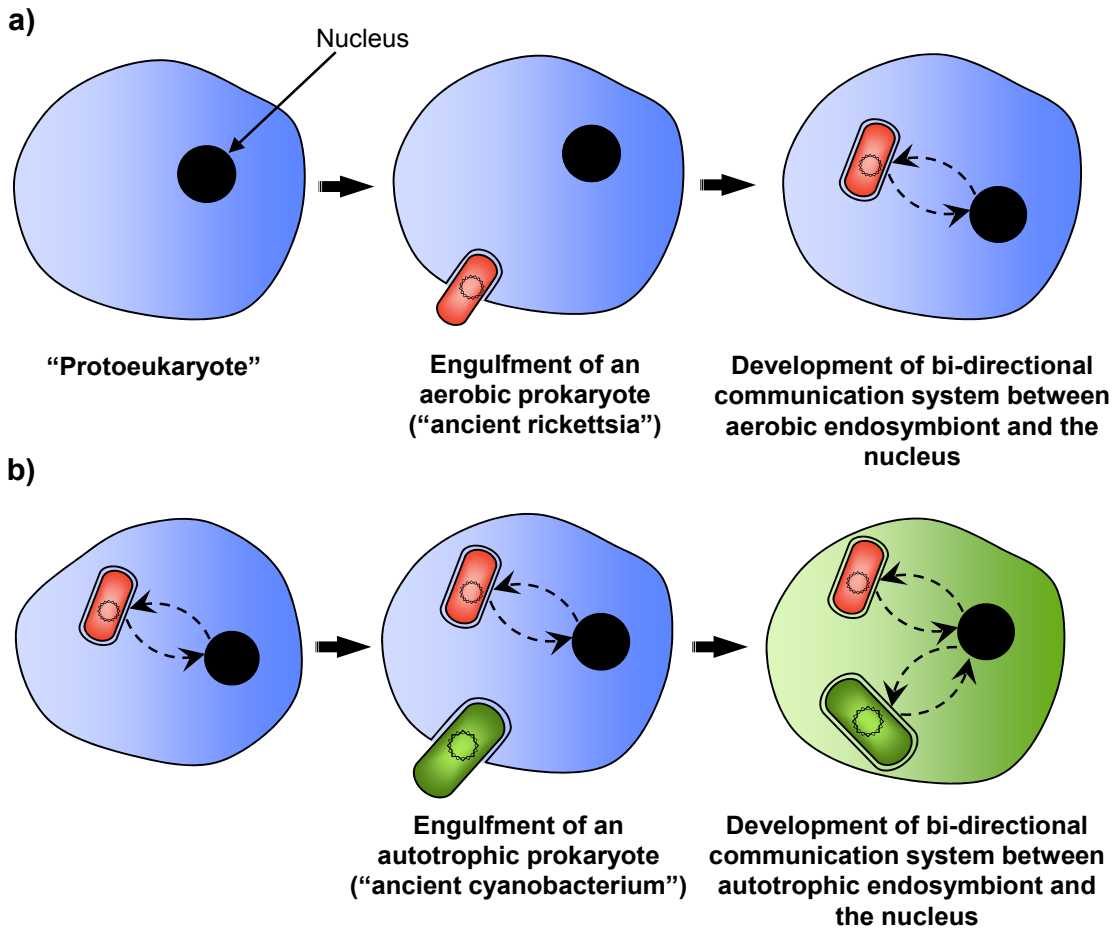

Figure 1.1. The origin of **a)** mitochondria and **b)** chloroplasts according to the Serial Endosymbiosis Theory (SET) and development of bi-directional communication systems. **a)** An aerobic or **b)** autotrophic prokaryotic cell was engulfed via endocytosis by a protoeukaryotic organism and instead of being digested it was kept as an endosymbiont, becoming a mitochondrion or chloroplast, respectively. In order for such an endosymbiotic consortium to survive the new organism had to develop an efficient bi-directional communication system (Beck 2001; Margulis 1970, 1993; Mereschkowsky 1905; Nott *et al.* 2006; Pfannschmidt 2010; Pogson *et al.* 2008; Whittaker and Margulis 1978).

chloroplast when fused with the transit peptide of the nuclear encoded small subunit of RuBisCO (Gatenby *et al.* 1988). Thus, even highly hydrophobic protein subunits can be transported from the cytoplasm to the chloroplast across its double envelope (Allen and Raven 1996).

Another hypothesis states that transfer of genes from the organelles is still taking place and it is just not yet completed (Gray 2000; Palmer 1997). Analysis of the chloroplast genome from the polyphyletic genus *Chlamydomonas* revealed the presence of genes such as *TSCA*, *TUFA*, *RP15*, *ORFA*, *ORFB*, or *ORF715* that have not been reported in any chloroplast DNA of the land plants (Boudreau *et al.* 1994; Boudreau and Turmel 1995), due to their transfer to the nucleus in the evolution process (Baldauf *et al.* 1990; Boudreau *et al.* 1997). However probable and at the same time difficult to verify, the hypothesis of gene transfer still being in process does not explain why the entire genome could not have been transferred. This is because of the non-random genes still remaining in the organelle of different groups of organisms (Martin *et al.* 1998) and also because of the ease with which this transfer could have taken place (Allen 2003b). The potential of such a transfer could be observed in *A. thaliana*, where the insertion of DNA with 99% sequence similarity to the entire mitochondria genome took place in the nuclear genome (Stupar *et al.* 2001).

Other hypotheses aimed to explain the retention of the chloroplast and mitochondria genomes like the "lock-in" (Bogorad 1975) or "frozen-accident" (von Heijne 1986) can be also excluded. The "lock-in" hypothesis states that the key subunits of the multi-subunit complexes must be synthesized *de novo* in a target compartment, otherwise their assembly might take place in other inter-cellular locations (Bogorad 1975). This hypothesis did not take into account uniparental, non-Mendelian inheritance of chloroplasts and mitochondria and separate from the nucleus replication of organellar DNA (Allen 2003a). The "lock-in" hypothesis can be also ruled out because of the present knowledge about mechanisms of protein targeting and import. The "frozen-accident" hypothesis claims that gene transfer stopped because of some kind of event like the origin of exocytosis and protein secretion (von Heijne 1986). According to this hypothesis, this could make precursors of the proteins encoded by the nuclear genes and targeted to the organelle indistinguishable from those synthesized for the export outside

the cell (von Heijne 1986). Yet considering the efficiency and precision of protein targeting it also does not seem to be the case.

Perhaps the most plausible explanation for retaining the genomes of chloroplasts (and mitochondria) is that co-location of genes with the product of their activity is necessary for efficient and direct regulatory coupling (Allen 1993a, b, 2003a; Allen and Raven 1996). This allows the redox control of chloroplast or mitochondrial gene expression which was shown to be a common feature in the case of proteins synthesized in these organelles (Allen 2003b). In other words, chloroplast and mitochondrial genomes contain genes for proteins involved in electron transfer that require rapid redox regulation (Allen 2003b).

The unicellular green alga *C. reinhardtii* has 72 protein encoding genes retained in the chloroplast (Maul *et al.* 2002) and 8 in the mitochondrion (Beck 2005). However, based on proteomic and bioinformatic studies, the chloroplast proteome is estimated to harbour from ~2000 to >3000 proteins (Abdallah *et al.* 2000; Leister 2003). Thus, the vast majority of the proteins needed for a functional chloroplast are encoded by the nuclear genome, translated in the cytosol and then transported into the chloroplast (Beck 2005). Maintenance of organelle genomes involves energy-consuming processes: over 90 proteins (e.g. ribosomal proteins, aminoacyl tRNA synthetases, and DNA and RNA polymerases) have to be encoded by the nuclear genome specifically for transcription and translation processes in the organelle (Alberts *et al.* 2002). Most of these proteins can be found exclusively in these organelles. Therefore, the nuclear genome has to hold about 90 genes simply to sustain the genetic systems of these organelles. Moreover, all of the regulatory mechanisms which are governed by proteins encoded by the nuclear genome, but required for efficient function of the chloroplast, must also be maintained.

1.3. Reactive oxygen species

1.3.1. Molecular oxygen chemistry

Molecular oxygen plays a key role in energy metabolism and respiration of aerobic organisms. Geologic records provide evidence for the oxidation of the Earth's surface by means of sulphate reduction (2.7-2.5 bya) or higher concentrations of

dissolved ferrous ion and banded iron formation sediments in the oceans of the Archaean and early Proterozoic (Canfield and Raiswell 1999). Yet, geochemical evidence suggests a delay of several hundred million years between the appearance of oxygen-evolving photosynthesis and the accumulation of significant amounts of oxygen in the atmosphere (Fennel *et al.* 2005). Fossil records suggest a rapid expansion of metabolism using oxygen in association with the evolution of oxygenic photosynthesis. This can be inferred, for instance, from algal diversification that parallels the ocean oxidation history deduced using sulphur isotopes (Anbar and Knoll 2002). The accumulation of oxygen to its modern level in the atmosphere and oceans, was very likely the key element that made the Cambrian Explosion possible (Barber 2004). According to studies on carbon and sulfur cycles, levels of atmospheric oxygen could have reached even up to 35% (14% higher than the present level) (Beerling *et al.* 1998) during the Carboniferous and Permian periods 375-275 mya (million years ago) (Berner 1999). Elevated levels of atmospheric oxygen could affect the metabolism of plants and animals and contributed to invasion of land by vertebrates, evolution of flight, or gigantism of insects (Berner 1999; Dudley 1998; Graham *et al.* 1995). However, the very nature of oxygen also makes it a possible toxic by-product of cellular metabolism. This is because of the formation of ROS which are highly active and can easily cause damage to living organisms by oxidation of various biomolecules.

Molecular oxygen in its ground state is a diradical species meaning it has two unpaired electrons with parallel spins (triplet state; Halliwell and Gutteridge 2007). This makes it idiosyncratic among elements and is the reason why it does not readily react with organic molecules (Halliwell and Gutteridge 2007; Krieger-Liszkay 2005). According to the Pauli Exclusion Principle, this feature precludes the possibility of reactions with a divalent reductant unless this reductant is also in a triplet state, i.e. matching that of oxygen. Thus, O_2 can only accept unpaired electrons, in other words oxygen reduction in biochemical reactions involves the transfer of only a single electron (Figure 1.2). Electrons removed as pairs from metabolites have to be passed to O_2 one at a time using conjugated coenzymes with stable radical oxidation states, such as flavin adenine dinucleotide (FAD) or metal containing cofactors (Voet and Voet 1990). Thus, while O_2 is normally quite stable, activation of O_2 can occur in two different ways:

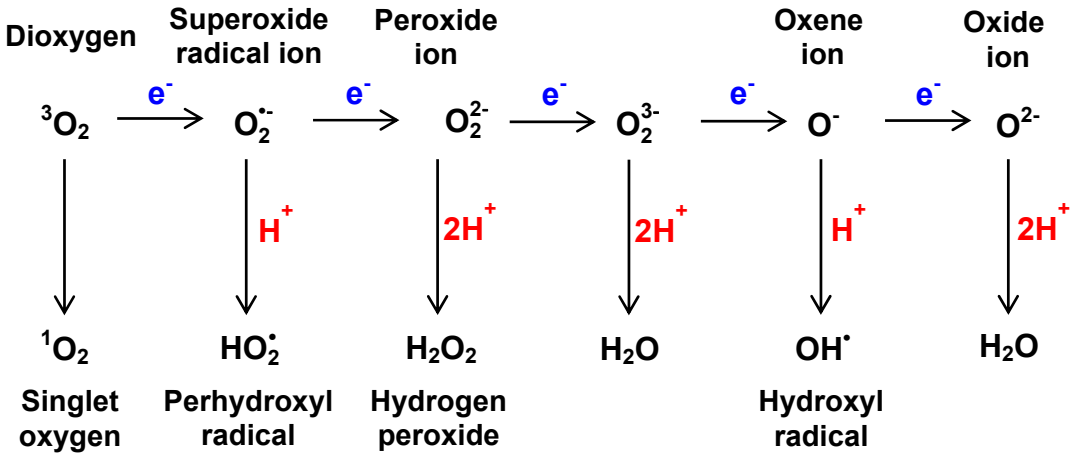

Figure 1.2. Ground state $^3O_2$ can be converted to ROS by energy transfer, leading to the formation of $^1O_2$ or by single electron reduction reactions. Sequential univalent reductions (one electron at a time) result in the generation of $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ (modified from Apel and Hirt 2004).

by absorption of energy adequate for reversing the spin of one of the unpaired electrons or by monovalent reduction. In the first case, if O_2 absorbs energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins. Unlike triplet oxygen, singlet oxygen ($^1O_2$) can participate directly in reactions involving two electrons – divalent reduction – thus making it highly reactive in the presence of organic molecules. The monovalent reduction of O_2 occurs in a stepwise fashion with the initial reduction producing superoxide anion ($O_2^{\bullet-}$), followed by hydrogen peroxide (H_2O_2). Superoxide anion can act either as an oxidant (e.g. in NADPH or ascorbic acid oxidation) or as a reductant (e.g. in reduction of cytochrome C or metal ions). Hydrogen peroxide can easily permeate biological membranes and that is why it is not compartmentalized in the cell. It was also demonstrated that plasma membrane aquaporins in plants conduct H_2O_2 (Dynowski *et al.* 2008). It can be used for example by peroxidases as a substrate in oxidation reactions in synthesis of complex organic molecules such as lignin or suberin (Arora *et al.* 2002; Quiroga *et al.* 2000). Hydrogen peroxide can also play a role in defence against pathogens in plants (Levine *et al.* 1994; Waetzig *et al.* 1999). Furthermore, the role of H_2O_2 in the induction of Programmed Cell Death (PCD) has also been well established (Desikan *et al.* 1998; Levine *et al.* 1996).

1.3.2. ROS sources in the cell

ROS can be produced in several ways in living organisms. Generally, they are formed as a by-product of biological redox reactions (Arora *et al.* 2002) in the mitochondria or chloroplast (Apel and Hirt 2004; Asada 2006; Buchanan *et al.* 2000; Halliwell 2006; Moller 2001). The electron transfer chain (ETC) in photosynthesis can generate $^1O_2$ or $O_2^{\bullet-}$ and it is the main point of origin of ROS produced in plant cells. During photosynthesis, electron transport produces O_2, NADPH, and ATP (Figure 1.3). Electrons are transferred from water to $NADP^+$. Inhibition of electron transfer from an excited chlorophyll molecule (Chl^*) that can be relatively long lived, can result in a process called intersystem crossing during which the electron flips its spin as it decays from the excited state, generating triplet state chlorophyll (^3Chl). ^3Chl is a diradical

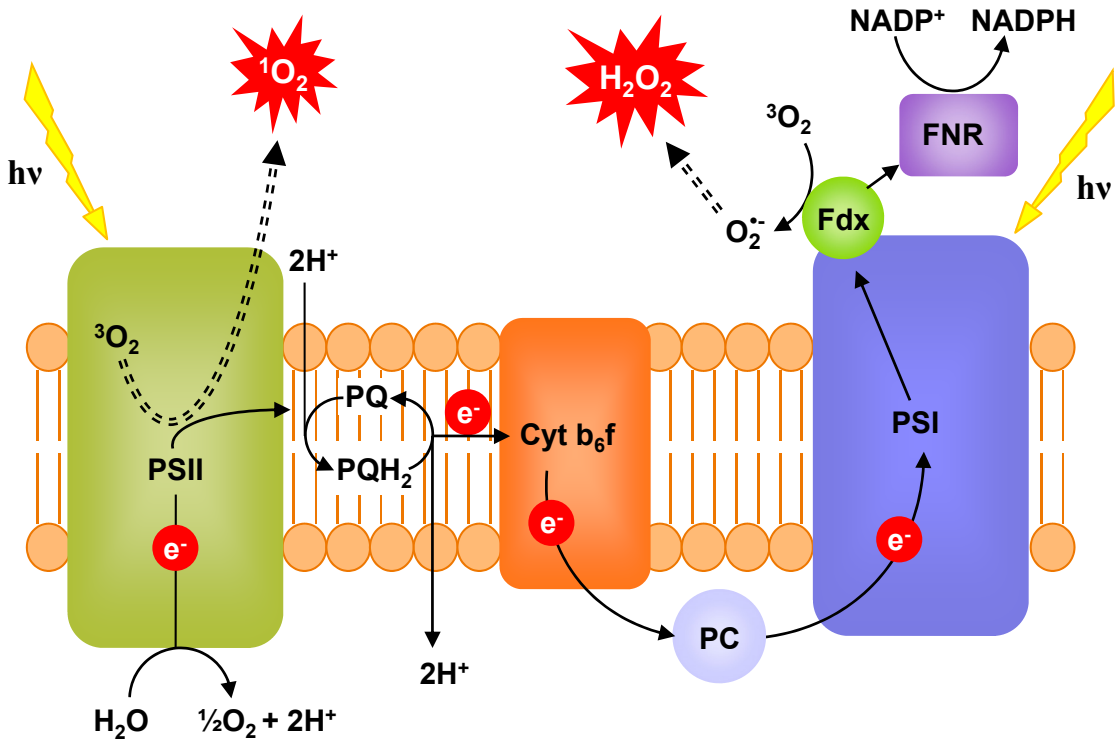

Figure 1.3. Reactive oxygen species ($^1O_2$, $O_2^{\bullet-}$, and H_2O_2) generation in the ETC of photosynthesis. Yellow arrows indicate photoexcitation of reaction center Chl. Black arrows highlight the electron flow used for carbon fixation. Doubled-dashed arrows indicate excess photon density for the capacity of the flux of the electron transport and thus ROS generation (modified from Asada 2006).

species that can react with O_2 to give $^1O_2$ (Figure 1.3). Thus, $^1O_2$ can be formed during photoinhibition, when plants under high-light intensities absorb more energy than can be used in photosynthesis. Such conditions can lead to oxidative damage, particularly to subunits D1 and D2 of the photosystem II (PSII) reaction center. One of the most potent generators of $^1O_2$ is P_{680}, the primary electron-donor chlorophylls in PSII.

Superoxide anion can be produced through a number of metabolic processes in the chloroplast. As a result of the Mehler reactions, $O_2^{\bullet-}$ is formed by PSI, where O_2 is reduced by the Fe-S center F_x of PSI (Asada 2006; Buchanan *et al.* 2000; Figure 1.3).Thus, an excess of electrons is transferred to oxygen by PSI leading to the formation of $O_2^{\bullet-}$ which is then converted to H_2O_2 by copper/zinc superoxide dismutase (Cu/Zn SOD; Figures 1.3 and 1.4). Formation of H_2O_2 can lead to severe damage such as lipid peroxidation, affecting chloroplast functions (Blokhina *et al.* 2003; Laloi *et al.* 2006; op den Camp *et al.* 2003; Reiss *et al.* 1983) and as a consequence triggering the down-regulation of some nuclear-encoded photosynthesis related proteins (Larkin and Ruckle 2008; Oelmuller and Mohr 1986; Pogson *et al.* 2008). To protect the chloroplast during the Mehler reaction, H_2O_2 is subsequently detoxified to water by the ascorbate peroxidase (APX) pathway (Figure 1.4). In this process electrons travel from water (electron source for PSII) to water (electron sink for PSI) and that is why the Mehler reaction is also called the water-water cycle or pseudocyclic electron transport.

While potentially damaging, the Mehler reactions (Mehler 1951) are critical for plants to protect themselves during photoinhibitory conditions. They maintain the electron flux through the photosynthetic apparatus even if there is not a sufficient amount of $NADP^+$ to act as an electron acceptor, otherwise CO_2 fixation is limited or inhibited (Asada 1999; Heber 2002; Makino *et al.* 2002). The Mehler reactions are also thought to help maintain a balance between NADPH production and ATP synthesis, because the electron flow allows proton transfer and subsequent ATP synthesis without a reduction of $NADP^+$. Excess superoxide radicals can also be formed by components of the electron transfer chain, such as PQ, the cytochrome b_6/f complex or PSI due to hyperreduction of electron carrier chains (Apel and Hirt 2004; Makino *et al.* 2002; Niyogi 1999). Thus, the production of ROS such as $O_2^{\bullet-}$ in PSI can be also a protective mechanisms, as long as

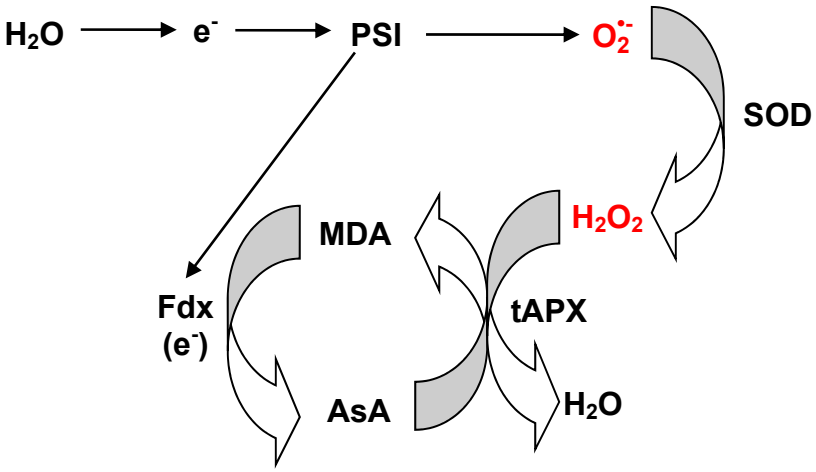

Figure 1.4. Water-water cycle (Mehler reaction). Superoxide radicals produced in photosystem I (PSI) are converted to H_2O_2, which is further eliminated by thylakoid-bound ascorbate peroxidase (tAPX). Activity of tAPX in the water-water cycle requires an ascorbate (AsA) as an electron donor. The AsA is regenerated in an ascorbate-monodehydroascorbate (MDA) cycle using electrons directly from the photosynthetic apparatus (modified from Mittler 2002).

ROS detoxification occurs at rates equal to the rate of ROS production (Asada 1999). It is only if ROS production exceeds detoxification that damage will occur. The proton gradient created during electron transport as part of the Mehler reaction contributes to ATP synthesis and enables de-epoxidation of violaxanthin to zeaxanthin and protonates light-harvesting complex (LHC) proteins in the thylakoid lumen (Horton *et al.* 1996; Neubauer and Yamamoto 1992). Protonated chlorophyll-proteins bind zeaxanthin and undergo conformational changes which turn them into energy-dissipating traps that compete with PSII for excitation energy and thereby protect PSII from photoinactivation (Heber 2002).

The activity of mitochondrial respiration is also a source of ROS in eukaryotic cells. Superoxide anion is produced by the autooxidation of reduced mitochondrial electron-transport components (Maxwell *et al.* 1999). For example, molecular oxygen can be directly reduced to $O_2^{\bullet-}$ in the flavoprotein region of NADH dehydrogenase, possibly by flavoprotein or iron-sulphur centre, or in the ubiquinone-cytochrome region of the respiratory chain by unstable semiquinone species (Arora *et al.* 2002).

1.3.3. ROS effects on cell biochemistry

Excess ROS production can lead to several types of damage in the plant cell. One of the most important forms of damage is lipid peroxidation (Halliwell and Gutteridge 2007). This process can be initiated by a hydroxyl radical ($OH^{\bullet}$) which removes a proton from a polyunsaturated fatty acid (PUFA) producing a lipid radical ($L^{\bullet}$). The product of this reaction reacts with molecular oxygen to produce a lipid peroxyl radical ($LOO^{\bullet}$), which in turn can abstract a proton from another fatty acid molecule forming a lipid hydroperoxide (LOOH) and another lipid radical (Catala 2006, 2010). In general, hydroxyl radicals are the most potent lipid oxidizing ROS (Mueller *et al.* 2006). Singlet oxygen can also react with unsaturated fatty acids. Direct reaction of $^1O_2$ with PUFAs leads to the formation of a complex mixture of LOOHs (Halliwell 2006; Mueller *et al.* 2006). These compounds can then participate in reactions that lead to the formation of products such as lipid aldehydes and alcohols. In the presence of metal catalysts hydroperoxides are unstable and can participate in Fenton reactions leading to the

formation of reactive alkoxyl and peroxyl radicals (Halliwell 2006). Lipid peroxidation causes changes in the structure and physical state of the membrane and its domains, which leads to rigidity and leakiness (Fischer *et al.* 2007a; Halliwell 2006). In the chloroplast, thylakoid membranes contain a large portion of polyunsaturated fatty acids (PUFA), thus making them especially prone to peroxidative damage (Baroli *et al.* 2003; Halliwell 2006; Vavilin *et al.* 1998). Overall, lipid peroxidation can significantly affect membrane functionality by: decreasing its fluidity, increasing permeability for compounds that normally would require transport through channels, inactivation of receptors and ion channels, damaging membrane proteins, and eventually causing the organelle or cell to rupture (Halliwell 2006). Noteworthy, the reactivity of $O_2^{\bullet-}$ and H_2O_2 is not sufficient to oxidize polyunsaturated fatty acids (Triantaphylides *et al.* 2008) and thus cellular membranes are not vulnerable to direct damage caused by these ROS. However, in the presence of Fe^{2+} both $O_2^{\bullet-}$ and H_2O_2 can be converted non-enzymatically to $OH^{\bullet}$ in the Fenton-type reaction (Halliwell and Gutteridge 2007).

ROS can also cause specific damage to proteins. Sulphur containing amino acids and enzymatic proteins with iron-sulphur centres are particularly sensitive to $O_2^{\bullet-}$ oxidation (Gardner and Fridovich 1991; Imsande 1999; Jang and Imlay 2010; Vranova *et al.* 2002). Thus, the Fe-S cluster proteins of the photosynthetic ETC are sensitive to ROS and damage to the cytochrome b_6/f complex and PSI results in decreased energy production due to a decrease in electron flow. This may lead to over-reduction of the PQ pool and subsequent oxidative damage due to photoinhibition of PSII (Imsande 1999; Kim *et al.* 2005; Velitchkova *et al.* 2003). Moreover, oxidation of the Fe-S clusters leads to the release of Fe^{2+} which may catalyze the conversion of H_2O_2 to $OH^{\bullet}$ by the Fenton or Haber-Weiss reactions (Halliwell and Gutteridge 2007; Imsande 1999; Vranova *et al.* 2002). As a result of ROS damage, some proteins undergo fragmentation of the peptide chain and many amino acid groups undergo specific irreversible modifications (Giustarini *et al.* 2004). Oxidation can cause protein aggregation as a result of cross bond formation (e.g. S—S, intermolecular cross-linking), altering electrical charge and increasing susceptibility to proteolysis (Farr and Kogoma 1991; Foyer and Noctor 2005a; Giustarini *et al.* 2004).

1.3.4. Defence mechanisms against ROS

A) Enzymatic antioxidative system

To protect themselves from oxidative damage, plants have developed enzymatic and non-enzymatic antioxidative systems. Antioxidative enzymes are directly involved in scavenging ROS (Apel and Hirt 2004; Asada 1999; Mittler 2002). Superoxide dismutase (SOD) catalyzes dismutation of $O_2^{\bullet-}$ to H_2O_2 (Asada 2006). There are three distinct types of SOD which are characterized based on the metal cofactor: Cu/ZnSOD – present in the cytosol, peroxisomes, and chloroplasts; MnSOD – present in mitochondria; and FeSOD – present in plastids (Bowler *et al.* 1994). The H_2O_2 produced by SOD is still toxic and must be converted to H_2O by catalases or peroxidases that utilize ascorbic acid and glutathione (GSH) as electron donors (Asada 1999). Catalases (CAT) are iron-heme-containing enzymes and can be found in the cytosol, glyoxysomes and peroxisomes, but not in the chloroplast (Willekens *et al.* 1997). Chloroplast H_2O_2 is removed by peroxidases, such as ascorbate peroxidase (APX), present in the cytosol and plastid (Asada 1999; Davletova *et al.* 2005). Glutathione peroxidases (GPX) which reduce H_2O_2 and more complex hydroperoxides and glutathione reductases (GR) which reduce oxidized glutathione are also found in the cytosol, mitochondria, and plastids (Asada 1999; Foyer and Halliwell 1976; Navrot *et al.* 2006). Although GPX are still classified as GSH-dependent, there is evidence indicating they have a higher affinity to thioredoxin (TRX) as the electron donor (Fischer *et al.* 2009; Iqbal *et al.* 2006; Navrot *et al.* 2006) and that is why it has been proposed to re-classify them as a novel class of peroxiredoxins (Herbette *et al.* 2007; Tanaka *et al.* 2005; see also *Section 1.2.4B*).

The role of these antioxidative enzymes in detoxification processes depends on their location in the cell or within the organelles. Perhaps that is why the overexpression of antioxidative enzymes did not always increase resistance to oxidative stress in earlier studies (Blokhina *et al.* 2003; Pitcher *et al.* 1991; Pradedova *et al.* 2011; Tepperman and Dunsmuir 1990; VanCamp *et al.* 1996).

B) Low molecular mass antioxidants

Non-enzymatic antioxidants are molecules that interact directly with oxygen radicals to dissipate their energy. Carotenoids prevent damage and provide detoxification by reacting with radical products and quenching the free radicals by terminating chain reactions. For instance, the energy associated with producing triplet state chlorophyll and hence $^1O_2$ formation, could be quenched by carotenoids in the antenna system where the edge-to-edge proximity between two molecules would be less than the van der Waals distance (3.6 Å) (Krieger-Liszkay 2005). However, the distance between carotenoids and triplet chlorophyll in the reaction centre is probably too large for direct quenching. Instead, the main function of β-carotene in photosynthetic tissue could be quenching of $^1O_2$ generated via the triplet state of P_{680} (Krieger-Liszkay 2005; Telfer 2002). Zeaxanthin and antheraxanthin formed from violaxanthin in the xanthophyll cycle participate in dissipation of thermal energy in the PSII antenna and thus work as another photoprotective mechanism (Gilmore 1997; Niyogi 1999).

Tocopherols are lipid-soluble molecules found mainly in the envelope of plastids where they are synthesized (Arango and Heise 1998), in thylakoid membranes (Fryer 1992), and in some cases in the plastoglobuli of the stroma where they are stored (Lichtenthaler *et al.* 1981). Tocopherols are comprised of the prenyl chain which anchors the molecule within the phospholipid bilayer (Niki *et al.* 1985) and the chromanol head oriented toward the water-lipid interface (Bisby and Ahmed 1989). In the thylakoid membrane of chloroplasts, the α-tocopherol (vitamin E) which is a predominate form found in green parts of plants, plays a role in scavenging $^1O_2$ generated during the quenching of the triplet state of the reaction center of PSII (Trebst *et al.* 2002). Tocopherols also help to stabilize membrane structure by removing oxygen free radicals or lipid peroxyl radicals (Foyer and Noctor 2005b; Munne-Bosch and Alegre 2002; Triantaphylides *et al.* 2008). Localization of the chromanol heads near the water-lipid interface and ability of tocopherols to diffuse laterally in the plane of the membrane allows it to react with peroxyl radicals. This position also allows tocopheroxyl radicals created in these reactions to be reduced by the water-soluble ascorbate or the glutathione in a tocopherols regeneration process (Munne-Bosch and Alegre 2002).

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphydryl group of cysteine (Rennenberg 1982). It can be found in the chloroplast, cytosol, mitochondria, peroxisomes and apoplast where it is capable to react with $^1O_2$, $O_2^{\bullet-}$ and $OH^{\bullet}$. However, its primary target of detoxification is H_2O_2 (Asada 1999; Mittler 2002; Noctor and Foyer 1998). Thus, the cysteine oxidation in GSH by H_2O_2 generates a thiyl radical that reacts with a second oxidized glutathione molecule, forming a disulphide bond (GSSG). Reduction of GSSG back to GSH is catalyzed by the enzyme glutathione reductase (GR, Figure 1.5). Glutathione helps stabilize membrane structure by eliminating fatty acid peroxides formed during lipid peroxidation. It also enables recycling of ascorbic acid from the oxidized form to the reduced form. Previous studies indicated that hydrogen peroxide is converted to H_2O by glutathione peroxidase using GSH as an electron source, and the oxidized glutathione is converted back to the reduced form by GR and NADPH produced by photosynthetic linear electron transport (Figure 1.5a; Mittler 2002; Nelson and Cox 2005). However, more recent studies suggest that glutathione peroxidases do not react with GSH, instead they use only reduced thioredoxin for their regeneration (Fischer *et al.* 2009; Iqbal *et al.* 2006; Navrot *et al.* 2006; see also *Section 1.2.4A*).

Ascorbate (AsA) is present in various cellular compartments but chloroplasts contain particularly high concentrations of this antioxidant (up to 20 mM; Noctor and Foyer 1998; Smirnoff and Wheeler 2000). AsA plays roles in many physiological processes such as light signalling, cell division, gene expression, cell death or pathogen responses (Arrigoni and De Tullio 2002; Foyer and Noctor 2011). It also plays a crucial role in plant defence against oxidative stress (Noctor and Foyer 1998). It is an acceptor of free radicals in reactions catalyzed by enzymes that protect the chloroplast from ROS oxidation. Ascorbate is the source of electrons in H_2O_2 reduction catalyzed by ascorbic peroxidase (APX). In this reaction AsA is oxidized to monodehydroascorbate radical (MDA) which is subsequently reduced back to ascorbate by reduced ferredoxin or chloroplastic MDA reductase. The MDA can be also spontaneously disproportionated to dehydroascorbate, followed by reduction of DHA to AsA by reduced glutathione (GSH) in a reaction catalyzed by DHA reductase (Figure 1.5b; Asada 1999; Mittler 2002; Noctor and Foyer 1998). Ascorbate is also a cofactor for enzymes such as violaxanthin

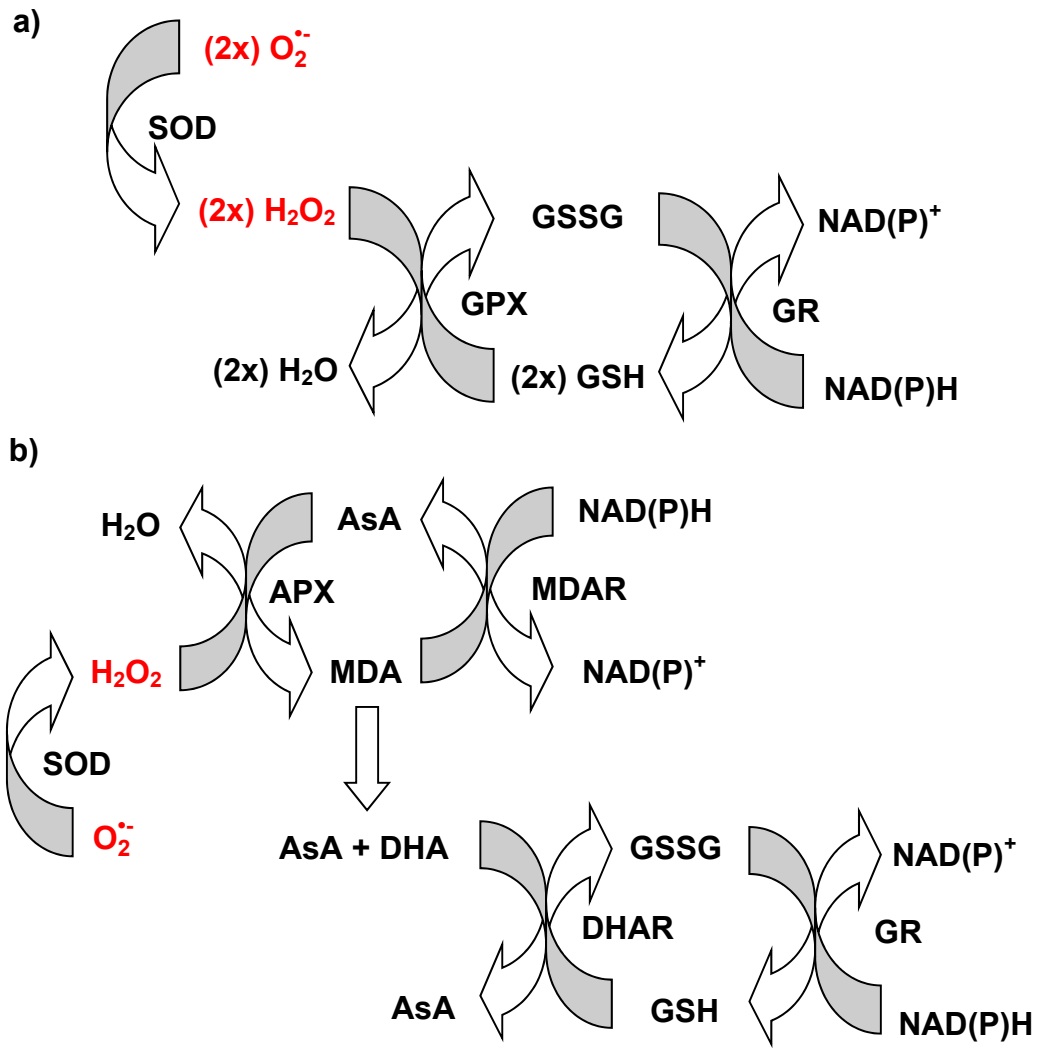

Figure 1.5. Systems for scavenging ROS in plant cells. **a)** The glutathione peroxidase (GPX) cycle. H_2O_2 is detoxified by glutathione peroxidase (GPX) which uses glutathione (GSH) as an electron donor in the formation of the oxidized form of glutathione (GSSG). GSSG is then regenerated to GSH by glutathione reductase (GR) which also utilizes electrons directly from NAD(P)H. **b)** The ascorbate-glutathione cycle. Superoxide dismutase (SOD) converts $O_2^{\bullet-}$ into H_2O_2 which is detoxified by ascorbate peroxidase (APX). APX requires ascorbate (AsA) as the electron donor. Ascorbate is then regenerated by monodehydroascorbate reductase (MDAR) via ascorbate-monodehydroascorbate cycle (MDA), utilizing electrons from NAD(P)H (modified from Mittler 2002).

deepoxidase, which is involved in xanthophyll cycle-mediated photoprotection (Smirnoff and Wheeler 2000), for enzymes required in the biosynthesis of plant hormones (Arrigoni and De Tullio 2002; Foyer and Noctor 2011; Mirica and Klinman 2008), and many other metabolic processes of the cell.

1.4. ROS signalling in plant cells

Following the endosymbiotic event, primitive eukaryotic cells needed to develop mechanisms for intra-cellular communication. Information needed to flow in both directions, i.e. from the nucleus to the organelle and vice versa. To fulfill this need, plants developed a complex system of signal recognition and transduction that relies on a network of physiological and biochemical interactions within the cell. The crucial elements of this system are intracellular Ca^{2+} and protein kinases. Ca^{2+} transduces many signals, playing the role of a second messenger within the cell. During signal transduction the Ca^{2+} concentration in the cytoplasm increases, initiating a signal transduction pathway often involving hundreds of proteins and other second messengers (Berridge *et al.* 2000; Trewavas and Malho 1998). Protein kinases belong to a highly diverse group of phosphotransferases present in all eukaryotes responsible for phosphorylation and thereby altering the activity of target proteins, and as a consequence affect regulation of responses to a variety of stimuli and control many cellular functions. Genes encoding protein kinases represent about 1-3% of the total number of functional genes in the eukaryotic genome and hundreds of them can be used in any particular event of signal transduction (Stone and Walker 1995). The net result of signal transduction and cross-talk, defined as components of a given signal transduction pathway interacting with a different signalling pathway, is determined by many various enzymes and proteins as well as second messengers in the cytoplasm and the plasma membrane. All of these compounds are involved in downstream reactions in many transduction sequences and constitute a network of great complexity.

In addition to the damaging effects of ROS, plants also appear to use them as second messengers in signal transduction processes (Apel and Hirt 2004; Baruah *et al.* 2009; Coll *et al.* 2009; Kim *et al.* 2009; Kim *et al.* 2008; Laloi *et al.* 2007; Meskauskiene *et al.* 2009; Moon *et al.* 2003; Orozco-Cardenas *et al.* 2001; Takahashi *et al.* 2011;

Vranova *et al.* 2002; Wilkins *et al.* 2011). Hydrogen peroxide can play the role of a signal-transducing molecule in the activation of defence responses in plants and an H_2O_2 cascade can interact closely with other signalling systems. It can mediate both intra- and extra-cellular communication during plant reactions to pathogens making it an important compound in systemic acquired resistance (Foyer and Noctor 2003). It is also a common secondary messenger in hormone-mediated events such as stomatal movement (Pei *et al.* 2000), cell growth (Rodriguez *et al.* 2002) and tropic responses (Joo *et al.* 2001). As mentioned above, under abiotic stress such as high-light, drought or extreme temperatures, both H_2O_2 and $^1O_2$ are produced in chloroplasts. In order for the plant cell to detoxify these ROS and repair any damage they cause, the appropriate enzymes and small antioxidants are needed at the appropriate level. Thus, stress reactions triggered by these abiotic stresses, can elicit a cellular response which up-regulates ROS defences via altered nuclear gene expression and post-translational regulation (Kim *et al.* 2008; Ledford *et al.* 2007; Reinbothe *et al.* 2010). Recent investigations suggest that ROS has a selective signalling effect in plants depending on its source and concentration (Galvez-Valdivieso and Mullineaux 2010; Karuppanapandian *et al.* 2011; Laloi *et al.* 2004; Pfannschmidt 2010).

It is unlikely that ROS such as H_2O_2 can be recognized as a classical signal molecule by a receptor protein, mainly due to the small size of this compound. However, the oxidising nature of H_2O_2 can be the source of direct chemical interactions (and thus signal propagation), for example thiol modifications in proteins (Cooper *et al.* 2002; Foyer and Noctor 2005a). This mechanism has been established for nitric oxide (NO) signalling, where this – similarly to H_2O_2 – small molecule reacts with the thiol side chain of Cys residues (*S*-nitrosylation) in stress signal transduction processes (Van Breusegem *et al.* 2008). Amino acids such as Tyr, Trp or His can also be oxidized and targets of ROS modifications. Furthermore, it was also suggested that proteins with a thiol group (-SH) can work as receptors for ROS (Foyer and Noctor 2005b; Hancock *et al.* 2006). The thiol group can be oxidized to sulphenic acid (-SOH) and further to sulphinic acid ($-SO_2H$) or sulphonic acid ($-SO_3H$). This oxidation depends on the mid-point redox potential of a given thiol group and its accessibility to the oxidant. In this

way proteins can be differentially controlled by fluctuations in the intracellular redox state – being regulated earlier or later as the redox state becomes more oxidized.

Modified –SH groups can also be re-reduced. Thioredoxins (TRXs) and glutaredoxin (GRXs) can act as protein disulphide reductases (Lemaire 2004; Schurmann and Jacquot 2000). Sulphinic acid groups can be reduced back to sulphenic acid by sulphiredoxins (found in *S. cerevisiae*; Biteau *et al.* 2003) and sulphenic acid groups can be reduced further by TRX or GRX to regenerate the thiol (-SH). Thiol groups can also be modified by the addition of other groups, for example glutathione (in a process called glutathionylation). Studies on *A. thaliana* revealed 79 polypeptides that can be modified in this way (Dixon *et al.* 2005). Such rapid and reversible ROS induced protein modifications can affect phosphorylation cascades or alter protein-protein, protein-lipid or protein-nucleic acid interactions making them excellent candidates for sensors and signals of ROS mediated events.

Following the application of exogenous H_2O_2 to *A. thaliana* suspension cultures, expression of approximately 170 genes increased more than 2-fold and expression of 65 genes was reduced (Desikan *et al.* 2001b) suggesting a role for H_2O_2 in regulation of gene expression. Hydrogen peroxide can also lead to activation of mitogen-activated protein kinases (MAP kinases), for example *A. thaliana* AtMPK3 and AtMPK6 (Desikan *et al.* 2001a; Kovtun *et al.* 2000) as well as MAPKKKs, ANP1 and OMTK1 (Kovtun *et al.* 2000; Nakagami *et al.* 2004). Thus, it appears that a high level of complexity exists in plant signalling processes, when examining responses to ROS.

However, questions arise with regards to the function of ROS in signal transduction pathways in the cell, primarily due to its reactivity. As mentioned above, $^1O_2$ and H_2O_2 can react with proteins, lipids or any organic compound in the cell. This feature can lead to damage to the cell but at the same time is a key element of how ROS affect other potential signalling mediators. Thus, there must be a dynamic balance of the concentration of ROS required to pass a signal to the target compound and enzymes preventing damage.

Levels of ROS below toxic concentrations are also able to trigger signalling cascades in bacteria. This was demonstrated in *Escherichia coli* where two main regulons are present that are responsible for protection against oxidative stress. Under H_2O_2 stress,

the *oxyR* regulon, through the OxyR protein, controls expression of at least 16 genes (Mukhopadhyay and Schellhorn 1997). The *soxRS* regulon is responsible for preventing damage from $O_2^{\bullet-}$ by inducing at least 15 genes encoding proteins such as superoxide dismutase or glucose 6-phosphate dehydrogenase (Demple 1999). Singlet oxygen was also shown to activate transcription of genes of the *soxRS* regulon. This finding came from *E. coli* treated exogenously with $^1O_2$ generated by thermodissociation of $NDPO_2$ (endoperoxide) which under thermal decomposition produces 3,3'-(1,4-naphthalidene) dipropionate and molecular oxygen, half of which is in the excited state (Di Mascio *et al.* 1989). This induction is also thought to be a protective mechanism against and triggered by oxidative stress (Agnez-Lima *et al.* 2001).

The function of ROS as signalling molecules has also been examined in *Rhodobacter sphareoides*. In this anaerobic, facultative-phototrophic bacterium, a transcriptional response to $^1O_2$ was shown to require the alternative ECF σ factor (extracytoplasmic function sigma factor, σ^E). σ^E factor levels increased under illumination efficient to excite the bacteriochlorophyll molecules in the presence of O_2 or the photosensitizer methylene blue. The σ^E factor was shown to be essential for a response to $^1O_2$ in carotenoid deficient mutants of *R. sphareoides* and thus to be involved in a $^1O_2$-dependent signalling pathway (Anthony *et al.* 2005).

C. reinhardtii demonstrated an acclimation response to ROS (defined as an enhanced ability to survive) after pre-treatment with sub-lethal levels of different ROS and then challenged with their higher concentrations (Ledford *et al.* 2007). Although several ROS were found to be able to trigger an acclimation response, such as hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BOOH) or metronidazole (MZ), the most dramatic acclimation was observed in the case of rose bengal (RB, a $^1O_2$ photosensitizer). The acclimation was shown to be relatively quick (after 30 minutes of pre-treatment), triggered by low concentrations of RB (0.25 μM), and induced transient changes in cell physiology that increased resistance to $^1O_2$ (Ledford *et al.* 2007). It was also shown to be specific to $^1O_2$ because only another $^1O_2$ photosensitizer, neutral red (NR), was able to induce a response resulting in higher tolerance to RB. Using microarray analysis Ledford *et al.* (2007) identified genes whose expression was increased (e.g. *GPXH*, glutathione peroxidase; *GSTS1*, glutathione-S-transferase; *TRXH*, cytosolic thioredoxin) or decreased

(*CAH1*, periplasmic carbonic anhydrase; *CCP1*, chloroplast envelope carrier protein) in response to $^1O_2$ pre-treatment (Ledford *et al.* 2007).

Interesting results have also come from the study of the conditional *fluorescent* (*flu*, because of the emission of strong red fluorescence upon an exposure to blue light) mutants of *A. thaliana*. The FLU protein is encoded by a nuclear gene and it is responsible for negative feedback control of chlorophyll synthesis (Meskauskiene *et al.* 2001). Thus, in the *flu* mutant the photosensitizer protochlorophyllide (Pchlide) accumulates in the dark and shifting etiolated seedlings to light results in Pchlide acting as a photosensitizer, with Pchlide undergoing intersystem crossing to the triplet state and then interacting with triplet molecular oxygen to produce $^1O_2$. This results in halted growth and development of necrosis. However, when kept under continuous illumination the *flu* mutant grows, matures, and produces seeds just like wild-type plants (op den Camp *et al.* 2003). Nevertheless, it was demonstrated that the expression of several genes was induced after shifting *flu* mutant plants from dark to light that seemed to be specifically activated as a result of $^1O_2$ generation, inducing a genetic program leading to cell death (op den Camp *et al.* 2003). Studies conducted on *A. thaliana flu* mutant plants provided tools to assess and differentiate between specific genetic responses, to $O_2^{\bullet-}$, H_2O_2, and $^1O_2$ generated in particular compartments (Dat *et al.* 2003; Queval *et al.* 2007; Rizhsky *et al.* 2003; Triantaphylides *et al.* 2008). These findings also indicated that ROS are not only highly-reactive damaging oxidizers but also specific signal-inducing molecules that activate stress-related responses (op den Camp *et al.* 2003).

1.5. Chloroplast-nucleus signalling pathways

Chloroplast development and function requires the coordinated expression of nuclear and chloroplast genes as well as the import of nuclear-encoded proteins from the cytoplasm. There is strong evidence that the chloroplast can regulate nuclear gene expression by signals originating in the plastid (Beale 2011; Beck 2001, 2005; Brown *et al.* 2001; Chan *et al.* 2010; Jung and Chory 2010; Pfannschmidt *et al.* 2003; Pogson *et al.* 2008; Figure 1.6). It is known that nuclear gene expression involved in photosynthesis

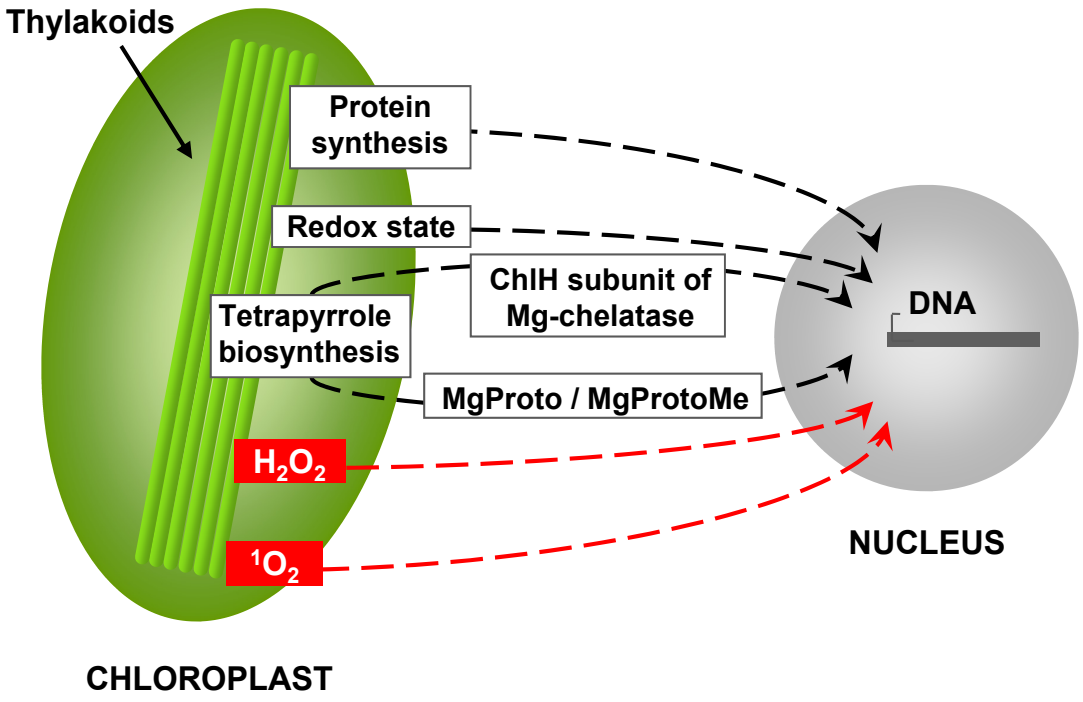

Figure 1.6. Hypothetical chloroplast-nucleus retrograde signalling pathways. Five separate signalling routes in communication between chloroplast and nucleus have been proposed: products of plastid protein biosynthesis; redox state of the photosynthetic ETC; intermediates of tetrapyrrole biosynthesis, accordingly to model 1 with the Mg-chelatase enzymatic complex sensing levels of Mg-porphyrins and its H subunit playing a key role in this system and/or model 2, with MgProto and/or MgProtoMe being directly involved in signalling cascades; chloroplast generated H_2O_2; and chloroplast generated $^1O_2$. Each effector may have possible positive or negative effects on transcription in the nucleus (modified from Beck 2005).

depends on the presence of a functional plastid (Koussevitzky *et al.* 2007; Mayfield and Taylor 1984; Nott *et al.* 2006), but what particular molecule(s) or mechanism(s) are involved is still unclear. It has been suggested that chloroplasts can exert retrograde control over nuclear genes by five signalling pathways (Figure 1.6): a) product(s) of plastid protein synthesis (Adamska 1995; Cottage *et al.* 2007; Gray *et al.* 2003; Pesaresi *et al.* 2006); b) intermediates of tetrapyrrole biosynthesis, in accordance to model 1 and/or model 2 (Beale 2011; Beck 2005; Chekounova *et al.* 2001; Kobayashi *et al.* 2011; Mochizuki *et al.* 2001; Strand 2004; Strand *et al.* 2003; Surpin *et al.* 2002; Voss *et al.* 2011; Zhang *et al.* 2011); c) chloroplast-generated $^1O_2$ (Galvez-Valdivieso and Mullineaux 2010; Krieger-Liszkay 2005; op den Camp *et al.* 2003; Wagner *et al.* 2004); d) chloroplast-generated H_2O_2 (Fryer *et al.* 2003; Galvez-Valdivieso *et al.* 2009; Karpinski *et al.* 1999; Mullineaux *et al.* 2006); e) redox state of the photosynthetic ETC (Braeutigam *et al.* 2007; Dietz and Pfannschmidt 2011; Escoubas *et al.* 1995; Pfannschmidt *et al.* 2009; Pfannschmidt *et al.* 2001; Pfannschmidt *et al.* 2003). These pathways are thought to participate in a complex signalling network that communicates the functional and physiological state of the chloroplast to the nucleus (Beale 2011; Beck 2005; Braeutigam *et al.* 2007; Chan *et al.* 2010; Jung and Chory 2010; Kleine *et al.* 2009; Nott *et al.* 2006; Pfannschmidt 2010; Pogson *et al.* 2008; Woodson and Chory 2008).

1.5.1. Plastid protein synthesis signalling pathway

There is evidence that treatment of plants with plastid-specific inhibitors of translation such as chloramphenicol, lincomycin, erythromycin or tagetitoxin (Rapp and Mullet 1991; Sullivan and Gray 1999) results in decreased expression levels of nuclear genes which encode proteins related to photosynthesis (Bajracharya *et al.* 1987; Gray *et al.* 1995; Oelmuller and Mohr 1986). However, the inhibitors were effective in preventing nuclear-gene expression only if applied within the first 2-3 days of seedling development. This suggests that the generation of a plastid signal must involve a product of early plastid gene expression during chloroplast development that becomes redundant at later stages. Other evidence also indicates that plastid protein synthesis generates a signal that is a prerequisite for the expression of nuclear genes encoding both plastid constituents and proteins found in other cellular compartments. However there is still a

lack of strong evidence on how the inhibition of protein synthesis in chloroplasts could affect nuclear gene expression or if it is really a product of plastid protein synthesis that is directly involved in chloroplast-to-nucleus signalling (Beck 2005).

Recently it was also suggested that peptide fragments from proteolysis of oxidatively damaged proteins in chloroplasts (as well as in mitochondria) can also act as signalling molecules in a retrograde chloroplast-to-nucleus signalling pathway induced by ROS (Moller and Sweetlove 2010). Because of the redox reaction network that takes place in the chloroplast there is a steady-state level of oxidized proteins present under normal growth conditions. Under stress conditions that result in higher ROS levels, the level of oxidation of many compounds, including proteins, increases (Davletova *et al.* 2005; Liu *et al.* 2008; Miller *et al.* 2007). There are also proteases present in the chloroplast (and mitochondria), responsible for cleavage of oxidized and/or damaged proteins (Adam *et al.* 2001; Kato and Sakamoto 2010). The main argument in the hypothesis of peptide fragments mediating ROS-induced signalling is that the ROS themselves lack specificity and thus cannot selectively regulate nuclear genes expression. However, their activity gives rise to specific protein modifications that depend on the type of ROS, the type of peptide and a compartment where they can come together (Moller and Sweetlove 2010). Hypothetically, the specific products of these reactions would be good candidates for mediating the signalling process because they would also include specific information, enabling them to regulate the expression of a certain cluster of genes. It was also postulated that the strength of the signal and therefore the level of gene expression would depend on the amount of different peptide fragments being able to avoid complete degradation by peptidases and thus to reach the nucleus (Moller and Sweetlove 2010).

1.5.2. Tetrapyrrole biosynthesis signalling pathway

The early steps of tetrapyrrole biosynthesis up to the formation of protoporphyrinogen IX (Protogen) take place only in the chloroplast. A fraction of Protogen is transported into the mitochondria to serve as the precursor of heme (Papenbrock and Grimm 2001). The other fraction remains in the chloroplast to serve as the precursor of both heme and chlorophyll. Protogen is transported to the plastid

envelope where it is oxidised into protoporphyrin IX (Proto) by the membrane-bound protoporphyrinogen IX oxidase (PPO; Joyard *et al.* 1998; Matringe *et al.* 1992). All subsequent steps leading eventually to the formation of chlorophyll in the thylakoid membrane take place in the plastid envelope, catalyzed by membrane-bound or membrane-associated enzymes (Joyard *et al.* 1990; Matringe *et al.* 1992; Reinbothe and Reinbothe 1996). Insertion of Fe^{2+} into ProtoIX by ferrochelatase leads to the formation of protoheme, the precursor of heme, while Mg^{2+} insertion into Proto by Mg-chelatase gives rise to Mg-protoporphyrin IX (MgProto), the precursor of chlorophyll (Beale 1999; Beck 2005; Strand *et al.* 2003).

In *A. thaliana* genomes-uncoupled (*gun*) mutants, *LHCB* and *RBCS* transcription is de-repressed in the absence of a functional chloroplast (Mochizuki *et al.* 2001; Susek *et al.* 1993). Thus, the *gun* mutants transcribe photosynthesis-related nuclear genes under conditions that normally result in the repression of these genes. Hence, the mutation acts to uncouple the transcription of *LHCB* from its dependence on the developmental state of the plastid. Analyses of these mutants provided evidence suggesting the involvement of compounds from the chlorophyll biosynthesis pathway in chloroplast-nucleus signalling (Figure 1.7). The *gun5* plants carry a mutation in the gene encoding the ChlH subunit of Mg-chelatase (Mochizuki *et al.* 2001). Thus it has been proposed that in addition to its catalytic role, ChlH has a signalling function. The plastid signal in this case is thought to be an altered porphyrin concentration sensed by ChlH and transmitted to the nucleus. Following treatment of *gun5* plants with norflurazon (a non-competitive inhibitor of carotenoid biosynthesis (Oelmuller 1989; Strand 2004), the mutation leads to impaired signal sensing by ChlH. Therefore, Lhcb transcription is de-repressed (Mochizuki *et al.* 2001; Rodermel 2001). Moreover, other nuclear photosynthetic genes, such as *RBCS* are also transcribed at higher levels in this mutant than in the wild-type. Studies of other *gun* mutant loci based on double-mutant analyses provided some evidence that *gun2*, *gun3* and *gun4* are on the same signalling pathway as *gun5* (Mochizuki *et al.* 2001; Vinti *et al.* 2000). *Gun1* probably affects a separate pathway for chloroplast-to-nucleus signal transduction, elements of which remain unknown (Strand 2004). However, more recent studies appear to contradict these hypotheses, as there does not appear to be any

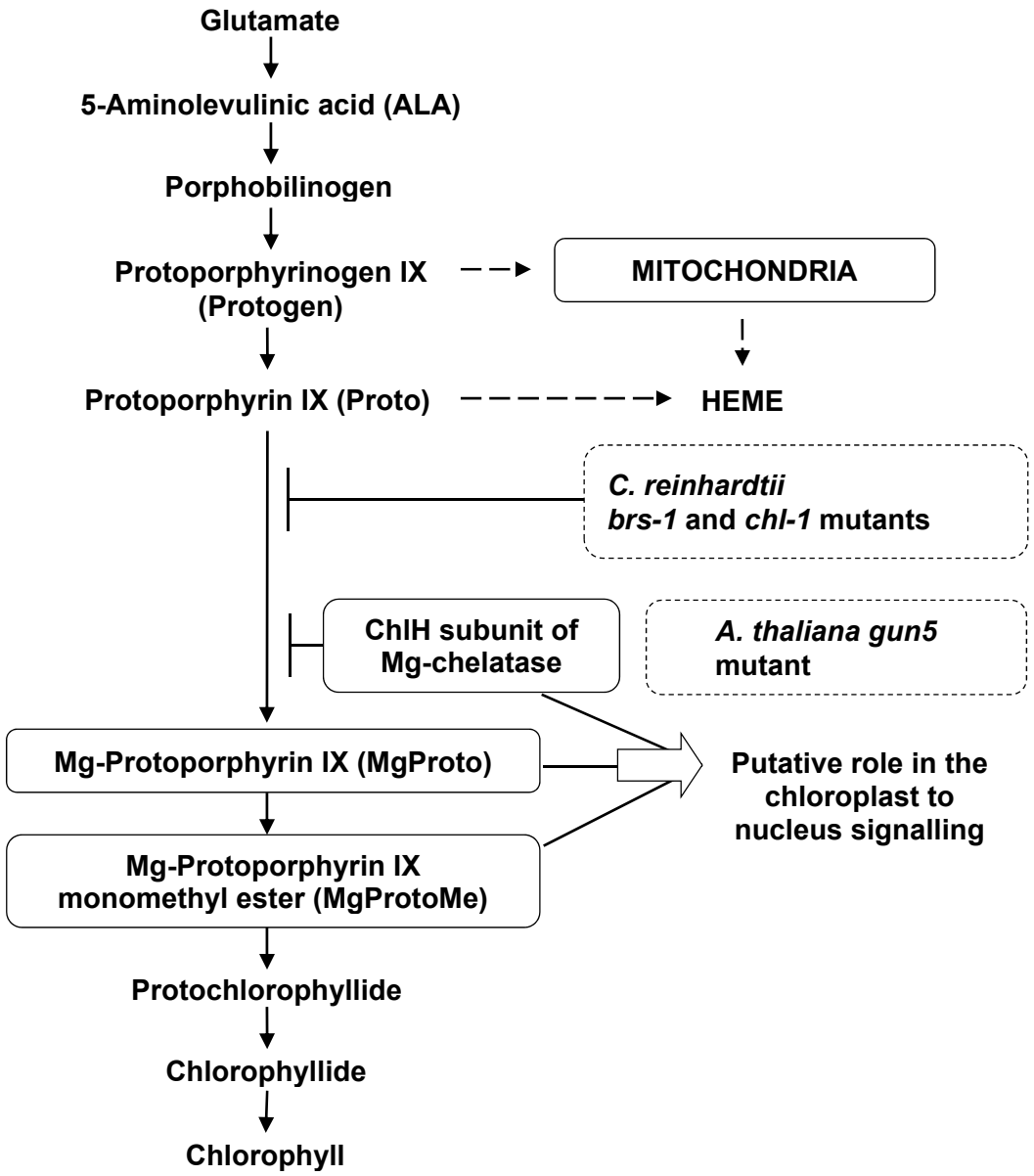

Figure 1.7. Chlorophyll synthesis pathway indicating mutants deficient in synthesis of the H subunit of Mg^{2+}-chelatase which is responsible for insertion of Mg^{2+} into protoporphyrin IX. *C. reinhardtii* chlorophyll deficient mutants *chl1* and *brs-1* (Chekounova *et al.* 2001; Wang *et al.* 1974) accumulate high levels of Proto and are deficient in the synthesis of MgProto and MgProtoMe. *A. thaliana gun5* mutant deficient in the synthesis of H subunit of Mg^{2+}-chelatase results in a subset of nuclear genes encoding chloroplast-localized proteins being uncoupled from the physiological state of the chloroplast, suggesting sensing and/or signalling function of H subunit of Mg^{2+}-chelatase (Beck 2001; Mochizuki *et al.* 2001; Rodermel 2001; Susek *et al.* 1993).

correlation between porphyrin levels and photosynthetic gene expression (Moulin *et al.* 2008).

Chlorophyll synthesis intermediates of *C. reinhardtii* have also been suggested to be involved in plastid to nucleus signal transduction pathways. Chlorophyll deficient mutants *brs-1* and *chl-1* (Chekounova *et al.* 2001; Wang *et al.* 1974) are responsible for the defect in synthesis of H subunit of Mg^{2+}-chelatase (Figure 1.7). This enzyme is responsible for inserting Mg^{2+} into protoporphyrin IX (Proto) in the chlorophyll synthesis pathway. Studies of these mutants revealed accumulation of Proto and a deficiency in the synthesis of further compounds of chlorophyll synthesis (MgProto and MgProto monomethyl ester, MgProtoMe; Figure 1.7). At the same time, these mutants (Beck 2001; Kropat *et al.* 1997) also exhibit a defect in expression of nuclear *HSP70A* and *HSP70B* chaperone genes. Induction of the expression of these nuclear heat shock genes was obtained by adding exogenous MgProto (Kropat *et al.* 1995). Thus, these two porphyrins (MgProto and MgProtoMe) are involved in the regulation of a specific subset of nuclear genes (Chekounova *et al.* 2001; Kropat *et al.* 2000). A similar effect was observed in intermediates of gametic differentiation called pre-gametes (Kropat *et al.* 1995). In these cells the synthesis of thylakoid-protein pigment complexes is reduced and neither accumulation of MgProto nor MgProtoMe is observed. However induction of *HSP70A* and *HSP70B* expression in pre-gametes could be also obtained by adding these two tetrapyrroles exogenously. These results suggest that in *C. reinhardtii* adding exogenous MgProto involved in chlorophyll biosynthesis can circumvent the block caused by mutation or induce expression of heat-shock protein in cells that do not normally express these genes in a specific developmental stage. Thereby, these data indicate participation of tetrapyrroles in the plastid to nucleus signalling pathway (Kropat *et al.* 2000). However, it is not clear whether the signals are due to tetrapyrroles themselves, or to the generation of $^1O_2$, a possible product of the interaction of light with these pigments (Beck 2001; Kleine *et al.* 2009; Moulin *et al.* 2008). In fact, it has been demonstrated that the *HSP70A* promoter contains specific elements that respond to $^1O_2$ and H_2O_2 (Shao *et al.* 2007) thus further complicating the analyses of these experiments.

1.5.3. Redox state signalling pathway

The activity of specific plant transcription factors may be regulated in response to changes in the redox milieu of particular components of the cell (Pfannschmidt *et al.* 1999). The redox-state of plastid ETC components is thought to be involved in signalling from the chloroplast (Brautigam *et al.* 2009; Pfannschmidt *et al.* 2003). The slowest step in the electron transfer from water to $NADP^+$ in the photosynthetic apparatus is the oxidation of the plastoquinone pool (PQH_2 to PQ) by the cytochrome b6/f complex. This feature and mediation in electron transport makes the plastoquinone pool a good candidate as a sensor of imbalanced redox states of PSII and PSI (Dietz and Pfannschmidt 2011). It has long been known that the redox state of the PQ pool can regulate state transitions. The thylakoid-associated kinase STT7 in *C. reinhardtii,* and its orthologue STN7 in *A. thaliana*, are thought to be responsible for LHCII phosphorylation which triggers the process of state transitions (Bellafiore *et al.* 2005). It has also been shown in *A. thaliana* that uneven excitation of the two photosystems can generate distinct signals originating from the PQ pool. These redox signals were shown to be responsible for regulation of the expression of specific nuclear genes (Fey *et al.* 2005; Wagner *et al.* 2008). Studies of the *A. thaliana* mutant *stn7* revealed that imbalanced excitation of the two photosystems can exert transcriptional changes which appear to be induced by signals from the PQ pool (Lemeille and Rochaix 2010). The redox state of the electron transport components also seems to be involved in regulation of photosystem stoichiometry, via translational or post-translational regulation of PSI synthesis, as was demonstrated in cyanobacteria (Fujita 1997).

Redox signals originating from the photosynthetic apparatus can also affect gene expression of the chloroplast genome. Those redox signals are responsible for affecting regulatory proteins involved in transcription, posttranscriptional modifications, as well as translational initiation (Pfannschmidt and Liere 2005). The plastid-encoded RNA polymerase is also controlled by signals originating from redox changes in the PQ pool via the thylakoid-associated kinase STN7 (Allen and Pfannschmidt 2000). STN7 may be a factor in further signal transduction activating a kinase network cascade that in turn controls phosphorylation of the sigma factor Sig1 (σ^1) regulating transcription of the reaction center genes of photosynthesis, namely *PSBA* encoding the D1 protein of PSII

and *PSAA/B* encoding the two central core proteins of PSI (Shimizu *et al.* 2010; Figure 1.8).

Studies of high-light acclimated cells of the green alga *Dunaliella* revealed stimulation of *LHCB1* transcription when shifted to low-light intensities (Escoubas *et al.* 1995; Maxwell *et al.* 1995) also suggesting a regulatory role for the redox poise of the plastoquinone pool. This was verified by the application of the specific PSII inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) that inhibits electron transfer from PSII to plastoquinone (PQ), keeping the PQ pool oxidized, resulted in enhanced *LHC* transcription, even under high-light conditions. However, partial inhibition of plastoquinol oxidation with DBMIB, which blocks electron flux from PQ to the cytochrome b_6/f complex ($cytb_6$/f), caused a repression of *LHC* expression at low-light intensities (Escoubas *et al.* 1995; Maxwell *et al.* 1995). Thus, redox signals may originate from $cytb_6$/f or PQ and play a regulatory role in nuclear gene expression. Studies with DCMU and DBMIB as well as analysis of $cytb_6$/f-defective mutants showed that redox signals can regulate transcription of PSI components (Pfannschmidt *et al.* 2001). Similarly, greater reduction of the ferredoxin (Fd) or the NADPH systems are also indicators of an excess of energy in redox reactions in the chloroplast (Dietz and Pfannschmidt 2011) and they could also play a role in retrograde signalling.

1.5.4. Hydrogen peroxide signalling pathway

In contrast to most ROS, H_2O_2 can diffuse across membranes and directly interact with signalling components outside the plastid. It is not known how cells can differentiate between H_2O_2 generated in plastids due to high-light conditions from H_2O_2 produced somewhere else (e.g. at the plasma membrane as a consequence of pathogen attack), however, H_2O_2 from the chloroplast can induce expression of the nuclear gene for ascorbate peroxidase (APX2). Infiltration of leaves with catalase resulted in reduced APX2 expression after high-light treatment (Beck 2005; Chang *et al.* 2004; Karpinski *et al.* 1999). A study using transgenic tobacco expressing a chloroplast-localized catalase or overexpressing a thylakoid-bound ascorbate peroxidase suggested that the role of H_2O_2 in the expression of a cytosolic ascorbate peroxidase is not exactly clear (Yabuta *et al.* 2004). How and where the plastid-generated H_2O_2 is sensed or how the plant cell can

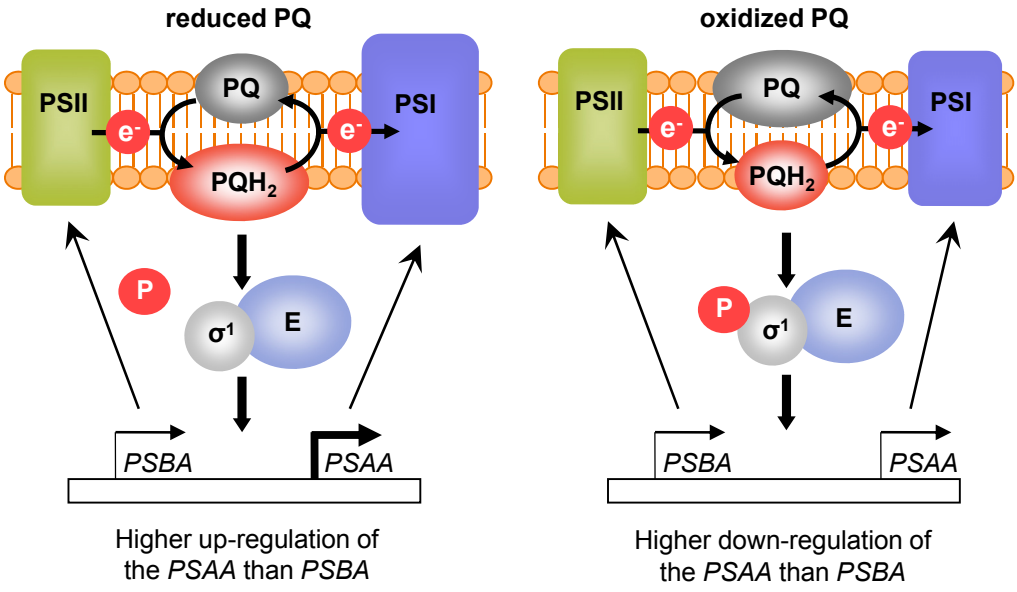

Figure 1.8. Model of the regulation of chloroplast gene expression by phosphorylation of the sigma factor (σ^1) in response to the redox signal originating in the PQ pool (modified from Shimizu *et al.* 2010). Different redox states of the PQ pool are the result of imbalanced excitation of the two photosystems (PSII and PSI), which in turn is caused by shifts from the light of a shorter wavelength (680 nm, higher energy light) to the light of a longer wavelength (700 nm, lower energy light). Under 680 nm light PSII is strongly active causing reduction of the PQ pool (PQH_2). This causes dephosphorylation of the sigma factor and up-regulation of the *PSAA* gene (encoding apoprotein of PSI) to induce PSI accumulation, balancing electron transport and thus preventing generation of ROS. Under 700 nm light, PSI becomes more active than PSII, yet its efficiency in general is much lower than PSI. Therefore, under these conditions the PQ pool (PQ) becomes more oxidized triggering phosphorylation of the sigma factor, and down-regulation of the *PSAA* gene (Munekaga *et al.* 2004). The enzyme (E) associated with the dephosphorylated sigma factor leads to high transcriptional activity for *PSAA* and *PSBA* but dephosphorylation of the sigma factor reduced expression of the *PSAA* more than it does in case of the *PSBA* as indicated by the size of the arrows denoting transcription (Shimizu *et al.* 2010).

differentiate the H_2O_2 signal generated under different conditions remains unidentified (Galvez-Valdivieso and Mullineaux 2010; Mubarakshina *et al.* 2010).

1.5.5. Singlet oxygen signalling pathway

Singlet oxygen has a very short half-life (about 200 ns) in the cell (Gorman and Rodgers 1992). As a result, the distance that it may travel was calculated to be approximately 10 nm, based on predicted diffusion rates (Beck 2005; Sies and Menck 1992). Its diffusion range is also limited due to its high reactivity with membrane lipids. This means that $^1O_2$ could play a specific role as an activator of genetically determined stress response program only if it is detected close to its source. One would predict that for chloroplast generated $^1O_2$ to have an effect outside of the organelle, it must be perceived in the chloroplast and its presence signalled to the cytoplasm and nucleus in order to alter mRNA accumulation.

As mentioned previously (*Section 1.3*), *C. reinhardtii* cells are able to acclimate to increased singlet oxygen exposure, through the altered expression of specific genes. However, transcriptional control does not appear to be the only target of the $^1O_2$-induced signalling pathways originating in the chloroplast. Involvement of $^1O_2$ in translational regulation was proposed in a study of the *tigrina (tig)-d.12* mutant of barley (*Hordeum vulgare*; Khandal *et al.* 2009; Reinbothe *et al.* 2010), which resembles the *flu* mutant of *A. thaliana* because it accumulates Pchlide in etiolated seedlings (Lee *et al.* 2003; Meskauskiene *et al.* 2001). Pchlide is a strong photosensitizer generating $^1O_2$ upon irradiation causing bleaching and death of the seedlings. However the $^1O_2$ produced in this process is not the direct cause of death, rather it is thought to act as a signal molecule activating stress-response pathways leading to apoptosis (op den Camp *et al.* 2003). Thus, in response to $^1O_2$ generated upon exposure of etiolated *tig-d.12* seedlings to light, transcript levels of photosynthetic genes did not change significantly compared to wild-type plants, while protein levels, such as RBCS (small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase), decreased significantly (Khandal *et al.* 2009). It was shown that upon dark-to-light shifts of the *tig-2.12* (Khandal *et al.* 2009) as well as the *flu* mutants (op den Camp *et al.* 2003), phosphorylation of the 80S ribosomal protein S6 declined (Reinbothe *et al.* 2010). Thus, it was proposed that a $^1O_2$-dependent up-

regulation of the *AtPK19* gene encoding one of the two S6 protein kinases in *A. thaliana* can act to provide a level of translational control in response to photooxidative stress (Reinbothe *et al.* 2010). It was shown that chloroplast-generated $^1O_2$-induced signalling that stimulates expression of a subset of nucleus-encoded genes different from those induced by H_2O_2 (op den Camp *et al.* 2003). Moreover, $^1O_2$-induced gene expression in *flu* mutants was shown to be antagonized by H_2O_2 and $O_2^{\bullet-}$ (Laloi *et al.* 2007).

Studies based on genetic suppressor screens of the previously mentioned *flu* mutant identified the chloroplast localized EXECUTER1 (EX1) and EXECUTER2 (EX2) proteins that are involved in mediating $^1O_2$-inducible nuclear gene expression (Lee *et al.* 2007). Although the mode of action of these proteins has yet to be elucidated, microarray analyses revealed several genes being induced in response to $^1O_2$. DNA microarray analyses of the mutants under high-light conditions indicated 70 genes that were specifically up-regulated and nine down-regulated (op den Camp *et al.* 2003). Because the *ex1* mutants were isolated as second site mutations, the EXECUTER1 protein is thought to be involved in perceiving $^1O_2$ as a signal or participating in the transduction of that signal. In all probability, $^1O_2$ can trigger different signalling pathways under different treatment conditions (Wagner *et al.* 2004). The *ex2* mutation was also identified as being involved in retrograde signalling induced by $^1O_2$ generated in chloroplasts. It was demonstrated that inactivation of both EX1 and EX2 in triple *ex1/ex2/flu A. thaliana* mutants abolished the up-regulation of most of the genes in response to $^1O_2$ (Lee *et al.* 2007).

Another example of involvement of $^1O_2$ in retrograde signalling comes also from *A. thaliana flu* mutants, where several suppressor mutations named $^1O_2$-linked death activator (*soldat*) have been identified that specifically abolish $^1O_2$-mediated cell death in *flu* mutant seedlings (Coll *et al.* 2009). A total of 19 *soldat* mutants of *A. thaliana* have been identified (Meskauskiene *et al.* 2009). For instance, the *soldat8*, carries a mutation in a gene encoding the SIGMA6 factor of the plastid-encoded RNA polymerase (PEP; Coll *et al.* 2009). The *soldat8* mutant is characterized by reduced non-photochemical quenching and enhanced sensitivity of the seedlings to light (Coll *et al.* 2009). The *soldat8/flu* double mutant seedlings, despite over-accumulation of the Pchlide in the dark, do not display the characteristic bleaching when shifted from dark to light (Coll *et al.*

2009). Moreover, when grown under very low-light conditions, the *soldat8* mutants seem to be more resistant to subsequent exposure to high-light conditions than wild-type plants (Coll *et al.* 2009). The acclimation in *soldat8/flu* double mutants, caused by disturbance in plastid homeostasis at the early stage of development, was suggested to be responsible for abolished $^1O_2$-mediated induction of cell death (Coll *et al.* 2009). However, unlike the *executer1* mutation (Wagner *et al.* 2004), the SOLDAT8 protein does not seem to impair $^1O_2$-mediated signalling, but rather appears to enhance light sensitivity via inactivation of the SIGMA6 subunit of the PEP and enhanced acclimation response (Coll *et al.* 2009). Among PEP-dependent genes analyzed, only *RRN16* encoding 16S rRNA and *PSBA* encoding D1 protein were affected at the transcript level. The D1 is core subunit protein of PSII and thus its impairment increases photoinhibition and $^1O_2$ generation. Because microarray and transcript analyses of possible impairment of plastid-encoded gene expression did not reveal any differences between *soldat8/flu* and the wild-type except *PSBA*, authors suggested that the light per se is a stress factor in seedlings development of *soldat8/flu* triggering the acclimation response (Coll *et al.* 2009). The reduced amount of the D1 in young soldat8 seedlings would lead to the enhanced production of $^1O_2$ at PSII even at light intensities that have no negative impact on the wild-type plants (Coll *et al.* 2009). This hypothesis is also supported by the chlorophyll fluorescence analysis where the wild-type plants subjected to photoinhibitory conditions (resulting in increased $^1O_2$ production) showed subsequent acclimation response to treatment with the high-light which was similar to that observed in *soldat8/flu* (Coll *et al.* 2009).

The *soldat10* mutation affects a plastid-localized protein related to a human protein containing a mitochondrial transcription termination factor (mTERF) motif (Fernandez-Silva *et al.* 1997). Based on the mTERF motif alignment using the SMART tool (http://smart.emblheidelberg.de/), at least thirty four other mTERF proteins are predicted to exist in *A. thaliana* (Meskauskiene *et al.* 2009), and a putative mTERF (MOC1) was found in mitochondria of *C. reinhardtii* (Schonfeld *et al.* 2004). *Soldat10* mutants display decreased levels of rRNA and protein synthesis which appears to affect chloroplast-nucleus signalling and nucleus-encoded gene transcript levels as well as causing increased light sensitivity (Meskauskiene *et al.* 2009). *Soldat10/flu* double mutants accumulate Pchlide in the dark but the $^1O_2$-induced cell death response is

abrogated at the seedlings stage of development (Meskauskiene *et al.* 2009). Interestingly, *soldat10* mutants retain $^1O_2$-induced nuclear gene expression, confirmed by examination of the transcript levels of genes known to be induced by $^1O_2$ in *flu* mutants, e.g. *BAP1* (*BONZAI1-ASSOCIATED PROTEIN*; op den Camp *et al.* 2003) or *ERF5* (*ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR5*; Danon *et al.* 2005). Thus, $^1O_2$-mediated retrograde signalling does not seem to be affected in *soldat10* mutants directly, rather the disturbed plastid homeostasis (impaired plastid gene expression possibly affecting ROS generation or the redox state of the plastid) seems to be responsible for suppression of the $^1O_2$-induced cell death response (Meskauskiene *et al.* 2009). However, the particular mechanisms of retrograde signals responsible for abrogated $^1O_2$-induced cell death response in *soldat10* still remain unidentified (Meskauskiene *et al.* 2009).

Using a *LUCIFERASE* gene fused to the promoter of an *AAA-ATPase* gene (*ATPases associated with various cellular activities*), which was shown to be specifically induced by $^1O_2$ (Laloi *et al.* 2006), second-site mutations have been identified in *A. thaliana flu* mutants after secondary mutagenesis with ethyl methanesulfonate (EMS), that either displayed activation of the reporter gene independent of $^1O_2$ (*constitutive activator of AAA-ATPASE*, *caa*) or insensitivity to $^1O_2$ (*non-activators of AAA-ATPASE*, *naa*; Baruah *et al.* 2009). Examination of these mutants suggests that the $^1O_2$-inducible retrograde signalling network does not operate in a linear isolated pathway but rather includes and interacts with other pathways of the intracellular signalling network (Baruah *et al.* 2009; Mittler *et al.* 2004; Overmyer *et al.* 2003). Thus, genes identified in second-site suppressor screens in *A. thaliana* may act pleiotropically, affecting the $^1O_2$-induced signalling stress responses as well as the control of plant development (Baruah *et al.* 2009).

In *C. reinhardtii* $^1O_2$-induced retrograde signalling was studied using $^1O_2$-generating photosensitizers NR or RB (Fischer *et al.* 2005; Leisinger *et al.* 2001). Based on DNA-microarray analysis it was demonstrated that application of these two photosensitizers induced different responses in terms of nuclear gene expression. While RB was observed to strongly and specifically induce only the *GPXH* gene (*GLUTATHIONE PEROXIDASE HOMOLOGOUS* of *C. reinhardtii*, also known as

GPX5), NR also induced a subset of genes expressed under more general oxidative stress conditions, accompanied by decreased accumulation of photosynthetic gene transcripts (Fischer *et al.* 2005). However, application of H_2O_2 or $O_2^{\bullet-}$-generating chemicals such as juglone, menadione or paraquat, only slightly induced expression of *GPX5* at sub-lethal concentrations. Experiments conducted with the organic peroxides *t*-BOOH or cumene hydroperoxide (CU-OOH), showed only limited levels of *GPX5* induction (Fischer *et al.* 2005; Leisinger *et al.* 2001). In response to RB, *GPX5* was the only gene out of 2700 genes tested by the DNA-microarray analysis that was strongly and specifically induced by $^1O_2$ (Fischer *et al.* 2005). These results, together with experiments where *C. reinhardtii* cells were exposed to photoinhibitory conditions, indicate that expression of *GPX5* (or *GPXH*) is specifically induced by endogenous or exogenously applied $^1O_2$ (NR or RB) and not by signals from disturbed photosynthesis or oxidative stress in general (Fischer *et al.* 2006; Fischer *et al.* 2005; Leisinger *et al.* 2001). Thus, it was postulated that a specific $^1O_2$ sensor must exist in *C. reinhardtii* that specifically induces expression of a small subset of genes, including *GPX5* (Fischer *et al.* 2005). However, so far no such sensor or signal transduction component has been identified. It was also proposed that $^1O_2$ generated in PSII can induce the expression of *GPX5* by diffusing out of the chloroplast (Fischer *et al.* 2007b). This conclusion was based upon the apparent detection of $^1O_2$ in the cytoplasm of *C. reinhardtii* using specific $^1O_2$ probes (Fischer *et al.* 2007b). The mechanism proposed would involve either direct diffusion of $^1O_2$ or a chain reaction process where $^1O_2$ leads to the formation of lipid hydroperoxides which decompose into peroxyl radicals with subsequent second generation of $^1O_2$ on the cytoplasm side of the chloroplast membrane (Fischer *et al.* 2007b; Miyamoto *et al.* 2007), and the presence of the $^1O_2$-sensing component in the stroma or cytoplasm (Fischer *et al.* 2007b).

Interesting results have also come from studies conducted on *C. reinhardtii npq1 lor1* double mutants that are deficient in lutein and zeaxanthin (Ledford *et al.* 2004), two carotenoids which play a critical role in photoprotection (Niyogi *et al.* 1997; Niyogi *et al.* 1998; see also *Section 1.2.4B*). Due to a lack of carotenoids, this double mutants have a high susceptibility to $^1O_2$ stress caused by exposure to photosensitizers or excess light (Ledford *et al.* 2004). High-light illumination followed by DNA-microarray analysis showed an increased expression of *GPX5*, *SMT1* (encoding C-methyltransferase), and

LI818r-1 (a stress-responsive member of the light-harvesting complex superfamily). These genes are associated with the early high-light acclimation response and an increase in their expression was observed in wild-type as well as in *npq1 lor1* double mutants (Ledford *et al.* 2004). However, a prolonged exposure to high-light conditions resulted in increased expression of *GPX5*, *LI818r-1*, *SMT1* along with *APX* and *FeSOD* specifically in the *npq1 lor1* mutants, while expression of these genes decreased to low-light levels in wild-type. The increase in expression of these genes in the *npq1 lor1* background was accompanied by decreased levels of the xanthophyll cycle pigment pool, α-tocopherol, β-carotene, as well as loss of chlorophyll, D1 and LHC proteins, leading eventually to cell death (Ledford *et al.* 2004). These results indicate the presence of a complex high-light-response system in *npq1 lor1* mutants and suggest a key role for $^1O_2$ as a signalling molecule mediating changes in gene expression involved in acclimation to excess-light conditions (Ledford *et al.* 2004).

The results from these different studies suggest that $^1O_2$ acts as a signalling molecule capable of exerting changes in nuclear gene expression through an inducible pathway. Some of the results indicate the possibility of $^1O_2$ signalling being mediated by the oxidation of membrane lipids (Girotti and Kriska 2004; Mueller and Berger 2009). On the other hand, results obtained by Fischer *et al.* (2007b) suggest that the $^1O_2$-signalling mediated compound is located in the aqueous phase of the chloroplast and it is very unlikely to be a lipophilic component (Fischer *et al.* 2007b).

The attempts undertaken so far to reveal mechanisms and effects of the chloroplast-nucleus retrograde signalling that involve ROS, or $^1O_2$ in particular, have revealed unexpected complication, often give paradoxical results and show the high complexity level and sophisticated cross-talk of this signalling network in plant cells.

1.6. *C. reinhardtii* as a model organism

Understanding the function and fundamental processes in eukaryotic cells has been facilitated due to developing technical advances and studying groups of relatively simple organisms. Progress in understanding mammalian biochemistry, genomics and cell function has been greatly accelerated by employing *Saccharomyces cerevisiae*. Yeasts are relatively simple, unicellular organisms, but their cellular function can be

generalized to a large extent to the function of the cells in more complex, multicellular organisms.

The green algae *Chlamydomonas reinhardtii* (Greek *chlamys*, a cloak; *monas*, solitary; Figure 1.9) plays a similar role in the study of plants, leading to it being called a "green yeast" (Goodenough 1992; Rochaix 1995). Like yeast, it is a unicellular organism with well understood haploid genetics. However unlike yeast it has both a chloroplast and flagella, leading to its use as an experimental system to study flagellar motility, basal body function (e.g. in studies of function and composition of centrioles), circadian clock mechanics (Matsuo *et al.* 2008), flagellar regeneration (Stolc *et al.* 2005), signal transduction, photosynthesis, and many biochemical or physiological processes (Keller *et al.* 2005; Lefebvre and Silflow 1999; Levine and Goodenough 1970; Li *et al.* 2004; Pazour *et al.* 2006).

Cultures of *C. reinhardtii* grow quickly with a doubling time of less than ten hours. Cell cultures behave homogeneously in terms of physiological and biochemical characteristics. They are haploid with a controlled sexual cycle, which allows tetrad analysis to be conducted. However, vegetative diploids can be forced, to allow dominance of mutations to be assessed. Because *C. reinhardtii* is a unicellular organism there is no tissue specificity nor are any hormones known to affect signalling biochemistry of the cell.

C. reinhardtii thus provides significant advantages for the genetic dissection of eukaryotic photosynthesis (Dent *et al.* 2005). Its photosynthetic apparatus is closely related to that of vascular plants and its nuclear (~120 Mb), chloroplast (~230 kb), and mitochondrial (~16 kb) genomes have been sequenced (Gray and Boer 1988; Gray *et al.* 2004; Maul *et al.* 2002; Merchant *et al.* 2007; Michaelis *et al.* 1990; Vahrenholz *et al.* 1993). All three genomes (nuclear, chloroplast, and mitochondrial) of *C. reinhardtii* have been sequenced and tools, such as transformation techniques, for genomics and molecular genetic studies have been developed. Unlike higher plants, photosynthesis is fully dispensable because cells can be grown heterotrophically in the dark using acetate as the sole carbon source, with cells still synthesizing chlorophyll and assembling a fully functional photosynthetic apparatus (Dent *et al.* 2001; Harris 2001). Thus, mutations affecting genes required for photosynthesis are not lethal but conditional.

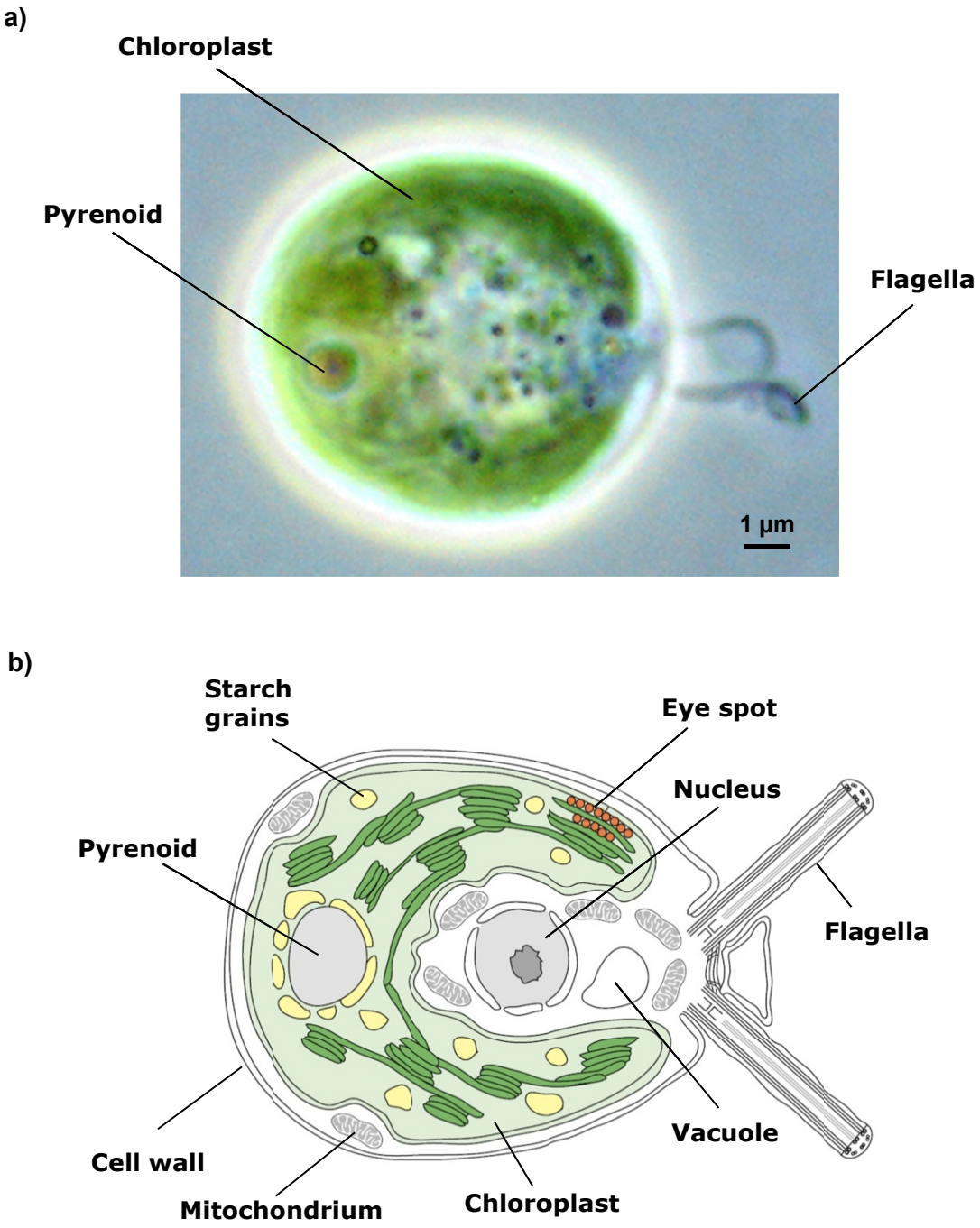

Figure 1.9. Overall view and ultrastructure of *C. reinhardtii* cell. **a)** *CC-3395* strain (~10 µm in diameter) as seen in ~680X magnification (400X Zeiss Axioskop 40 microscope magnification and ~1.7 digital camera zoom); **b)** ultrastructure of the wild-type *C. reinhardtii* cell (modified from Dent *et al.* 2001).

Previously, some limitations in studying *C. reinhardtii* resulted from codon usage bias (favouring G and C in the third position) and the resulting GC richness of its nuclear DNA. This has made it difficult to express foreign DNA, such as antibiotic resistance or reporter genes (Sizova *et al.* 2001; Stevens *et al.* 1996). However, modern techniques such as artificial gene synthesis and codon-adapted reporter genes (e.g. GFPs or luciferases) or reporter constructs based on endogenous DNA have provided tools for efficient transformation, selection, and expression of the DNA introduced into nuclear, chloroplast or mitochondrial genomes of *C. reinhardtii* (Franklin *et al.* 2002; Fuhrmann *et al.* 1999; Lumbreras and Purton 1998; Lumbreras *et al.* 1998; Matsuo and Ishiura 2011; Mayfield and Schultz 2004; Sizova *et al.* 2001; Stevens *et al.* 1996). Additionally, many mutant strains of *C. reinhardtii* are publicly available from the Chlamydomonas Center (http://www.chlamy.org/strains.html) providing material to study many important biological functions.

Thus, because of the several advantageous characteristics of *C. reinhardtii* and development of efficient molecular techniques "green yeast" also provides a good model system to study signalling processes that occur between organelles. This includes the indispensable bilateral communication system between chloroplast and nucleus. This study focuses on retrograde signalling, induced by $^1O_2$, generated in the chloroplast and attempts a genetic dissection of the components involved in mediating these signalling processes.

1.7. Thesis objectives

The main goal of this research was to investigate aspects of $^1O_2$ signalling using the unicellular algae *C. reinhardtii* as a model system. The primary goal was to develop a robust reporter strain which could be used to perform a secondary mutagenic screen for colonies that were deficient in $^1O_2$-dependent signalling. This screen was subsequently used to identify the genetic components involved in retrograde signalling from the chloroplast to the nucleus triggered by $^1O_2$ stress.

The first objective was to develop a reliable monitoring system that would allow the monitoring of changes in nuclear gene expression due to $^1O_2$ production in the chloroplast or due to the application of the photosensitizers rose bengal or neutral red.

The hypothesis was that using an engineered reporter gene consisted of the well characterized $^1O_2$-dependent 5' RR of the *GPX5* gene fused to a promoterless version of the *ARS2* reporter gene, would allow to reproducibly observe the $^1O_2$-dependent control of nuclear gene expression. This system has been used in the past (Fischer *et al.* 2007a; Fischer *et al.* 2006; Fischer *et al.* 2004; Leisinger *et al.* 2001) but was refined in the present study to produce a reliable, stably transformed *C. reinhardtii* strain that could be used as the basis for examining $^1O_2$-dependent signalling through a mutagenic screen.

The second of the objectives was to use the *GPX5-ARS2* reporter strain to perform a secondary mutagenic screen in an attempt to identify strains in which the $^1O_2$-dependent signalling pathway did not elicit the appropriate response to application of $^1O_2$ photosynthesizers. The hypothesis was that random mutagenesis of the nuclear DNA would disrupt gene(s) encoding components involved in mediating $^1O_2$-inducible signalling and thus would allow selection of mutant lines which would provide biological material for identification of the mutated genes.

Subsequently, the third objective was to identify the disrupted gene(s) from mutant strains generated by secondary mutagenic screen. The hypothesis was that using a DNA marker for an insertional mutagenesis would make possible identification of the disrupted gene(s) and thus components involved in $^1O_2$-dependent retrograde signalling, and by these means would provide needed insight into this process in *C. reinhardtii*.

In the remaining chapters, the approach to test formulated hypotheses will be described and a working model of how one particular protein may be involved in the $^1O_2$-dependent regulation of *GPX5* expression will be presented.

CHAPTER 2.
DEVELOPMENT OF A *C. REINHARDTII* STRAIN TO MONITOR THE $^1O_2$-DEPENDENT CHLOROPLAST-NUCLEUS SIGNALLING PATHWAY

2.1. Introduction

Glutathione peroxidases (GPX) are a group of enzymes present in both animal and plant cells (Criqui *et al.* 1992; Depege *et al.* 1998; Drotar *et al.* 1985; Holland *et al.* 1993; Leisinger *et al.* 1999; Roeckel-Drevet *et al.* 1998; Ursini *et al.* 1995). They reduce H_2O_2 and organic peroxides to water and the corresponding alcohol (Fischer *et al.* 2009). There are two main classes of GPX identified so far. One class includes enzymes that contain selenocysteine (SeCys) in their active sites and use glutathione (GSH) as an electron donor (Ursini *et al.* 1995). The other class of GPXs do not contain SeCys (NS-GPX) and is the only class of GPXs found in higher plants (Herbette *et al.* 2007). It was shown that all plant NS-GPX show higher activity using thioredoxin (TRX) rather than GSH as the electron donor (Navrot *et al.* 2006).

Both SeCys-containing and NS-GPXs capable of metabolizing organic peroxides as well as H_2O_2 have been identified in *C. reinhardtii* (Dayer *et al.* 2008; Leisinger *et al.* 1999; Yokota *et al.* 1988). The *GPX-HOMOLOGOUS* gene (*GPXH*) used in this study was initially named *GPXH*, but was renamed *GPX5* by the genome sequencing consortium. It belongs to the NS-GPX group and is one of three GPXs of this type identified in *C. reinhardtii* (Dayer *et al.* 2008). Using the *GPX5* upstream regulatory region fused to an arylsulfatase reporter gene it has been shown that *GPX5* is transcriptionally up-regulated in a specific manner by $^1O_2$ photosensitizers (Leisinger *et al.* 2001). Thus, a *GPX5* 5' regulatory region fused to *ARS2* could be used as a $^1O_2$-responsive reporter gene.

Based on the work of Leisinger *et al.* (2001), a prediction was made that cells transformed with the *GPX5-ARS2* would respond by producing the purple/brown chromogenic product, characteristic of ARS2 enzymatic activity, when exposed to conditions which increase the production or accumulation of $^1O_2$. Higher levels of $^1O_2$ can be generated by application of a type I photosensitizer NR or a type II photosensitizer

RB. In type I reactions, the excited photosensitizer reacts directly with the substrate or transfers an electron or hydrogen atoms leading to the formation of the semi-reduced radical form of the substrate (Björn 2008; Marks *et al.* 1984; Figure 2.1). On the other hand, the type II reaction involves generation of $^1O_2$ when a photosensitizer, after absorption of light goes from the excited singlet state to a triplet state, and transfers the energy to oxygen in triplet state, generating singlet oxygen (Björn 2008; Foote 1991; Figure 2.1). In general, photosensitizers such as NR or RB absorb light and decay energetically via intersystem crossing to the triplet state. As mentioned above the triplet organic molecules can then interact with oxygen (Figure 1.2) to yield $^1O_2$. Additionally, both NR and RB photosensitizers have been shown to specifically induce *GPX5* by increased generation of $^1O_2$ (Fischer *et al.* 2004; Leisinger *et al.* 2001; Leisinger *et al.* 1999). However, while $^1O_2$ generation by RB is a result of energy transfer from triplet state photosensitizer to $^3O_2$, the mechanism of $^1O_2$ generation by NR is not exactly clear. One possible explanation is an inhibiting effect of NR on photosynthesis. It was demonstrated that low concentrations (up to 50 μM) of NR can reduce photosynthetic electron transfer from H_2O to PSI by 50-60% (Opanasenko *et al.* 2002). This includes pH-dependent inhibition of PSII observed only at pH 8.5 but not at pH 6.5-7.5, PQ inhibition with parallel stimulation of basal electron transport at PSI (Opanasenko *et al.* 2002).

Examination of O_2 evolution in isolated spinach thylakoid membranes and in intact *C. reinhardtii* cells treated with 20-40 μM NR indicated inhibition of photosynthetic activity by 10-45% (Fischer *et al.* 2004). The inhibiting effect of NR on the photosynthetic apparatus was also supported by chlorophyll fluorescence experiments. Photosynthetic activity in dark-adapted *C. reinhardtii* cells treated with NR or RB indicated a strong reduction (by 89%) of the F_v/F_m parameter even at 1 μM NR when compared to untreated samples, while no effect from RB was observed in the dark (Fischer *et al.* 2004). The $^1O_2$ generation by increasing concentrations of NR and RB was also equated to isolated thylakoid membranes exposed to different intensities of white light illumination, using electron paramagnetic resonance (EPR) spin trapping. While both RB or NR treatments resulted in increased $^1O_2$ generation in a light-intensity-dependent manner, RB produced $^1O_2$ both in aqueous solution and in the presence

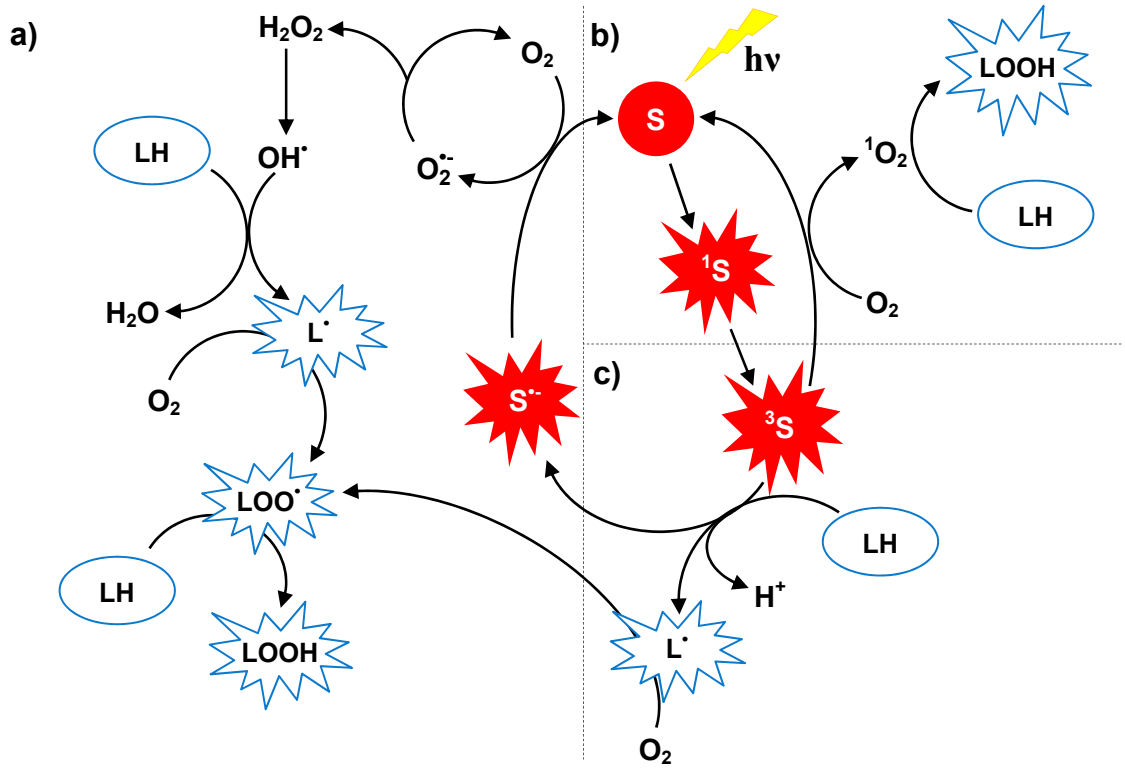

Figure 2.1. Two types of photosensitizing reactions. An example of how a pigment (S) upon excitation by light (hν) goes from an excited state (^{1}S) to an excited triplet state (^{3}S) causing damage to a membrane lipid (LH). **a)** In a type I reaction, an oxygen-dependent hydrogen abstraction, an electron is transferred to O_2 creating $O_2^{\bullet-}$ which via formation of H_2O_2 and $OH^{\bullet}$ abstracts a hydrogen atom from the lipid creating a lipid radical ($L^{\bullet}$) which after combining with ground state oxygen produces a lipid peroxy radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ can initiate a chain reaction by abstracting an atom from another lipid molecule producing a lipid peroxide (LOOH). **b)** In type II reactions, a pigment in the triplet state may react with O_2 to form $^1O_2$ which can directly produce LOOH. **c)** In a classical type I reaction, the pigment in the triplet state abstracts the hydrogen atom from the lipid, which also leads to its degradation to $L^{\bullet}$ and consequently to $LOO^{\bullet}$, and LOOH, initiating similar chain reaction (modified from Björn 2008).

of thylakoids (Fischer *et al.* 2004). However, NR at concentrations below 20 μM did not produce $^1O_2$ in aqueous solution although an increase in the $^1O_2$ level was observed in the presence of thylakoids treated with 5 μM NR (Fischer *et al.* 2004). Therefore, it is possible that oxidative damage caused by NR, e.g. lipid peroxidation of the thylakoid membranes increases the probability of backwards reactions and charge recombination in PSII, and as a result increased $^1O_2$ generation. This idea is supported by the effect of NR on photosynthesis-associated nuclear genes (PhANGs) expression (Fischer *et al.* 2005). The response to NR treatment correlated rather to more specific treatments such as RB, as in the case of *GPX5*, or dark-light shifts and *HSP80*, rather than to the general oxidative stress response (Fischer *et al.* 2005). Thus, the conclusion can be drawn that induction of *GPX5* expression seems to be an effect of increased generation of $^1O_2$ due to an inhibition by NR in the photosynthetic apparatus (Fischer *et al.* 2006; Fischer *et al.* 2004, 2005).

In this chapter, the construction of a single vector containing a *GPX5-ARS2* reporter gene and a positive selectable marker will be described. Following the successful transformation of *C. reinhardtii* cells, the effect of applications of different ROS, organic peroxides, photosynthesis inhibitors, and light stress on the expression of the engineered reporter gene will be analyzed. The hypothesis is that higher ROS production, specifically $^1O_2$ generation either via exogenous application or produced endogenously, will induce expression of the reporter gene which can be monitored.

2.2. Materials and methods

2.2.1. C. reinhardtii strains and culture conditions

C. reinhardtii CC-3395 is a cell wall deficient (*cwd*), arginine auxotroph (*arg 7-8*), and mating type minus (*mt$^-$)* strain. *CC-3395* was obtained from the Chlamydomonas Center (University of Minnesota, St. Paul, MN, USA) and it was used for the initial mutagenesis. Wild-type strain 6C$^+$ (mating type plus) was obtained from the laboratory of J.-D. Rochaix (University of Geneva, Geneva, Switzerland).

Cultures were grown on solidified agar (BioShop Canada Inc., Burlington, ON, Canada) medium or in liquid medium of Tris-acetate-phosphate (TAP) (Andersen 2005;

Gorman and Levine 1965; Harris 1989). The chemical composition of the TAP media is provided in Table A6.1. Because the *CC-3395* strain carries a mutation in the structural gene *arg7*, which encodes argininosuccinate lyase, making it an arginine auxotroph, the arginine was added to the media at a concentration of 2.5 mg L^{-1}.

For inducing higher photosynthetic activity, cells were grown on solidified agar or liquid Tris-phosphate (TP) media (without the acetate as a reduced carbon source); in this case pH was adjusted by hydrochloric acid (EMD Chemicals, Gibbstown, NJ, USA). All lines were grown under continuous cool white-light illumination provided by fluorescence tubes (Cool White Ecolux Rapid Start, 30 W, F30T12/CW/RS/ECO; General Electric, Fairfield, CT, USA) at 50 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (growth-light, GL), at 23 °C, and in case of liquid cultures on an orbital shaker (130 rpm).

For the selection of transformed colonies, cells were grown on solidified TAP containing 1% (w/v) agar supplemented with the appropriate antibiotic (10 μg mL^{-1} paromomycin (Sigma-Aldrich, Oakville, ON, Canada), 15 μg mL^{-1} zeocin (Invitrogen, Carlsbad, CA, USA), 10 μg mL^{-1} hygromycin B (Invitrogen) or 10 μg mL^{-1} kanamycin (EMD Chemicals)).

2.2.2. DNA isolation

For DNA isolation, cells were grown in TAP medium, in flasks on an orbital shaker (130 rpm), under GL, at 23 °C. After reaching the mid-log phase of 3-5 x 10^6 cells mL^{-1}, cells were harvested by centrifugation at 3000 x g for 5 min, at 22°C and DNA extraction was performed as follows. The cell pellet was resuspended in 1 mL cold buffer A (10 mM Tris-HCl pH 8.0. 10 mM EDTA, 10 mM NaCl). Cells were centrifuged and washed additionally two times with the same cold buffer A. After the last wash the cells were resuspended in 250 μL of cold buffer A. Twenty five microliters of protease K (1 mg mL^{-1}; Omega Bio-Tek, Norcross, GA, USA) and 25 μL 10% sodium dodecyl sulfate (SDS, EMD Chemicals) were added and the samples were precipitated with 1 mM final concentration of NaCl and 2 volumes of cold 95% ethanol, at -70 °C for 15 min. After precipitation samples were centrifuged for 5 min at 4 °C, washed twice with cold 70% ethanol, and air-dried. Samples were resuspended in 100 μL of H_2O and 2 μL of RNaseA

(1 mg mL^{-1}) was added, followed by incubation at 37 °C for 30 min. After incubation, 50 µL of 7.5 M ammonium acetate were added allowing proteins and polysaccharides to precipitate on ice for 10 min. Samples were centrifuged for 30 s, the supernatant was treated with 300 µL cold 95% ethanol, and samples were placed at -70 °C for 15 min, followed by centrifugation at 21,250 x g, at 4°C, for 10 min. After air-drying, samples were resuspended in 100 µL H_2O and DNA was precipitated with 200 mM NaCl and 300 µL 95% ethanol, followed by centrifugation at 21,250 x g, at 4°C, for 10 min. The pellet was washed twice with 70% cold ethanol, allowed to air-dry, and DNA was resuspended in 50 µL H_2O.

2.2.3. Molecular cloning

Escherichia coli DH10B strain was used for routine cloning experiments, following standard lab protocols (Sambrook and Russel 2001). Electrocompetent *DH10B* were prepared following growth in Terrific Broth (TB) medium without NaCl (to reduce the number of cell washes in competent cells preparation) accordingly to a standard protocol (Surzycki 2000) and stored at -80ºC.

All PCR products were examined by electrophoresis in ethidium bromide (EtBr, 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, Sigma-Aldrich)-containing 1% (w/v) agarose (EMD Chemicals) gel. Purification of DNA fragments from agarose gel was performed using GeneJET Gel Extraction Kit (Fermentas, Canada Inc., Burlington, ON, Canada), EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, ON, Canada) or Silica Bead DNA Gel Extraction Kit (Fermentas).

T4 DNA ligase containing kits for cloning purposes were obtained from Fermentas (GeneJET PCR Cloning Kit, InsTAclone PCR Cloning Kit, CloneJet PCR) or from Invitrogen (Zero Blunt PCR Cloning Kit).

All bacteria transformations were conducted by electroporation in 0.2 cm gapped cuvettes (VWR International, Mississauga, ON, Canada), using a Bio-Rad Gene Pulser II Electroporation System, at capacitance 25 µF, resistance 200 ohms, and 2.5 kV pulse (12.5 kV cm^{-1}). After electroporation bacteria were transferred to LB agar containing appropriate antibiotic for selection of transformants.

Plasmid DNA cloned in recombinant *E. coli* cultures was isolated using the GeneJET Plasmid Miniprep Kit (Fermentas) or EZ-10 Spin Column Plasmid DNA MiniPreps Kit (Bio Basic Inc.). Cloned products were verified by sequencing at National Research Council of Canada – Plant Biotechnology Institute (NRC–PBI, Saskatoon, SK, Canada).

2.2.4. GPX5-ARS2 reporter vector construction

With the long term goal of using reporter strains to perform a secondary mutant screen, the reporter gene was combined with a selectable marker to yield a single transformation vector system. This system where *ARS2* and $Paro^R$ are on the same plasmid was designed to provide a more efficient transformation system because co-transformation with an additional plasmid containing an antibiotic resistance cassette was not needed. This system also minimized damage to the genome of the transformed cells because only one insertion event is required.

For a positive control, to ensure the ARS2 system is functional as previously reported, the 5'-RR of the gene encoding the PSAD subunit of the photosystem I, which is constitutively expressed under regular growth conditions (Fischer and Rochaix 2001), was used to drive *ARS2* expression. The pSL18 plasmid (Depege *et al.* 2003) carries the *PSAD* promoter aligned in a reverse orientation in respect to the *AphVIII* gene (encoding the paromomycin resistance cassette) fused to the 5'- and 3'-untranslated regions of the *C. reinhardtii RBCS2* gene, and additionally 5'-region of *HSP70A* as a second promoter, creating a system which provides an effective dominant selectable marker for *C. reinhardtii* (Sizova *et al.* 2001). The fragment carrying both *AphVIII* and the *PSAD* 5'-RR was excised with *KpnI* and *EcoRV* restriction digestion from the pSL18 plasmid and inserted into the corresponding sites of the pJD54 plasmid (Davies *et al.* 1992) which carries a copy of the *ARYLSULFATASE 2* gene (*ARS2*) with the 5' regulatory region removed. This cloning step resulted in the generation of the pPB3 vector carrying the *ARS2* gene under the control of the *PSAD* 5'-regulatory region but in reverse orientation with respect to the *AphVIII* selectable marker (Figure 2.2a).

To create a $^1O_2$-dependent reporter strain, genomic DNA was isolated from a wild-type strain of *C. reinhardtii* ($6C^+$), as previously described, and was used as a

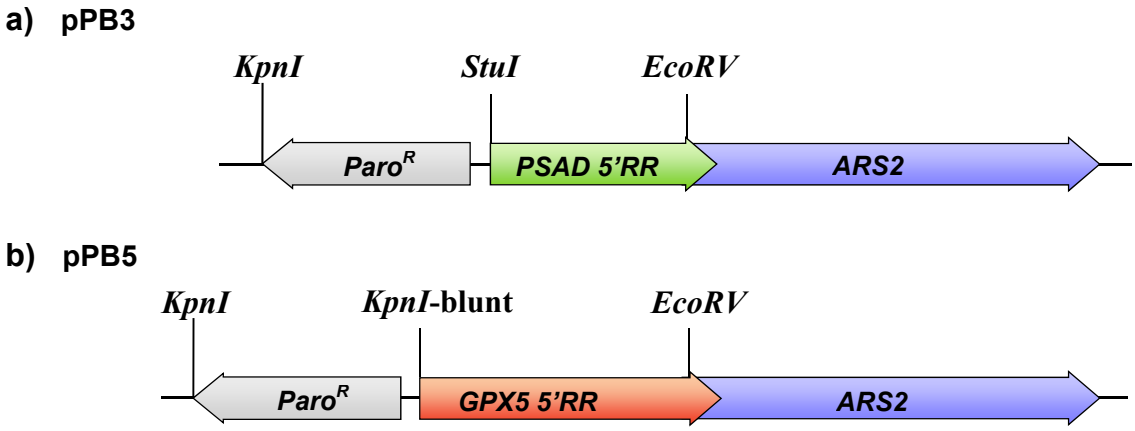

Figure 2.2. Maps of constructs used for *C. reinhardtii* transformation. *GPX5* 5' and *PSAD* 5' regulatory regions (5' RR) were fused independently with the *ARS2* gene and paromomycin ($Paro^R$) resistance cassette. Thus, both *PSAD-ARS2* (**a**) and *GPX5-ARS2* (**b**) reporter genes have been engineered in the same plasmids with a paromomycin resistance cassette, producing pPB3 and pPB5, respectively.

template for the PCR amplification of a 1362 bp fragment of the *GPX5* gene (Leisinger *et al.* 2001). The oligonucleotides KW64 and KW65 (Table A6.2) used for the *GPX5* RR amplification were designed to include 5' *KpnI* and 3' *EcoRV* endonuclease restriction sites, respectively. The PCR was conducted using a High Fidelity PCR Enzyme Mix (Fermentas), following the manufacturer's instructions with the 10X High Fidelity PCR Buffer, dNTP Mix (0.2 mM each; Fermentas), and $MgCl_2$ concentration adjusted separately to 3 mM. All reactions were performed using the iCycler thermal cycler (Bio-Rad) with the following reaction conditions: initial denaturation at 94ºC for 3 min, 35 cycles of 94ºC for 30 s, 54ºC for 30 s, and 72ºC for 1 min 30 s and a final extension at 72ºC for 10 min. The *GPX5* 5' fragment was blunted using T4 DNA Polymerase (Fermentas) and ligated into pCR-Blunt vector (Invitrogen) following the manufacturers' instructions and used to transform by electroporation electro-competent *DH10B E. coli.* Plasmid DNA was isolated from *E. coli*, examined by EtBr-stained 1% (w/v) agarose gel electrophoresis, and sequenced (NRC–PBI) to ensure that the correct fragment was obtained and no errors were introduced during the PCR amplification process. The *GPX5* 5' RR fragment was excised using *KpnI* and *EcoRV* enzymes (Fermentas), followed by blunting of the overhanging 3'-*KpnI* using T4 DNA Polymerase (Fermentas). The pPB3 plasmid was digested with *StuI* and *EcoRV* to remove the PSAD 5' RR, followed by insertion of the blunt GPX5 5' fragment, the resulting vector was called pPB5 (Figure 2.2b). The *GPX5* 5'fragment was tested for directionality by PCR and sequencing (NRC–PBI). The *GPX5-ARS2* expression cassette in pPB5 and the *PSAD-ARS2* cassette in pPB3 were oriented in reverse direction to the $Paro^R$ promoter to avoid the possibility of read-through from the $Paro^R$ promoter.

2.2.5. Generation of the C. reinhardtii $^1O_2$-dependent reporter strain

Cultures of *C. reinhardtii CC-3395* strain were grown to a cell density of 2-5 x 10^6 cells mL^{-1} (250 mL) in TAP in Erlenmeyer flasks, under GL (50 μmol photons m^{-2} s^{-1}). Colonies were harvested by centrifugation (1500 x g, for 5 min, at 22ºC) and cells were resuspended in TAP supplemented with 60 mM sucrose, concentrating the cell density to 5 x 10^8 cells mL^{-1}. Transformation was performed with pPB5 (*GPX5-ARS2*) and pPB3 (*PSAD-ARS2*) linearized with *KpnI* endonuclease (Fermentas). Independent

transformations with both plasmids were carried out using 1 μg of plasmid DNA in an aliquot of 250 μL of cells in 0.4 cm gapped electroporation cuvettes (VWR). Prepared cuvettes were kept on ice for 30 min. Electroporation was performed using the Bio-Rad Gene Pulser II Electroporation System (Bio-Rad, Mississauga, ON, Canada) at the following settings: capacitance 25 μF, resistance 200 ohms, and 800 V pulse (2 kV cm^{-1}). Following electroporation, cells were transferred into 10 mL of TAP supplemented with 60 mM sucrose (in 15 mL Falcon tubes) and left for 16 h to recover on an orbital shaker (130 rpm) under GL, at 23ºC. After the recovery period, cells were harvested by centrifugation (2000 x g, for 3 min), resuspended in 500 μL TAP, and spread on TAP agar containing 10 μg mL^{-1} paromomycin and 2.5 mg L^{-1} arginine. This allowed selection of successfully transformed cells which were then transferred onto TAP agar supplemented with 2.5 mg L^{-1} arginine for further growth.

2.2.6. Induction of GPX5-ARS2 and arylsulfatase assay

Arylsulfatase is a periplasmic enzyme, synthesized during sulfur-limited growth in *C. reinhardtii*. It is almost completely secreted into the culture medium by cell wall deficient cells (De Hostos *et al.* 1988). Its activity can be assayed using 5-bromo-4-chloro-indolyl sulfate (X-SO_4; Davies *et al.* 1992), nitrophenyl sulfate (NP-SO_4; Davies *et al.* 1992; De Hostos *et al.* 1988), and α-naphthyl sulfate (N-SO_4) as chromogenic substrates (Ohresser *et al.* 1997). Neither ARS mRNA nor ARS enzyme activity can be detected in untransformed cells grown in sulfur-sufficient medium (De Hostos *et al.* 1989).

To detect arylsulfatase activity *in situ*, colonies grown on TAP plates (*Section 2.2.1*) were picked up using a sterile toothpick in roughly the same amount and were spotted on solidified agar containing TAP or TP (supplemented with 2.5 mg L^{-1} arginine) with addition of 10 μM NR (Fisher Scientific, Ottawa, ON, Canada), 10 μM RB (Sigma-Aldrich), 10 μM DCMU (Sigma-Aldrich), 0.1 mM *t*-BOOH (Sigma-Aldrich) or 1 mM H_2O_2 (Sigma-Aldrich) into the media, prior to agar solidification, to test the inducibility of *GPX5-ARS2*. The NR and RB were dissolved in water and 5 mM stock solutions were stored at 4°C in the dark. DCMU was dissolved in 95% ethanol as a 10 mM stock solution and was stored at 4°C. However, the final concentration of ethanol in the

DCMU-treated samples did not exceed 0.095% (v/v). The H_2O_2 used in this study was stored at 4°C and was diluted with H_2O to 100 mM before use. The *t*-BOOH was stored at 4°C and was diluted with H_2O before use.

Chemical treatment of *C. reinhardtii* transformants was performed in combination with exposure to dark, moderate-light (ML, 100 µmol photons m^{-2} s^{-1}) or high-light conditions (HL, 920 µmol photons m^{-2} s^{-1}) for 16 hours (Davies *et al.* 1992) in a temperature-regulated environment (air-conditioned room, air circulation facilitated by an additional fan system). After exposure to experimental conditions no apparent differences in cell growth between tested lines were observed and because of the clearly pronounced ARS2 activity, the differences in the growth rate were assumed not to be affecting the results and thus, were not taken into account. Additionally, controls without the addition of NR, RB, DCMU, *t*-BOOH or H_2O_2, were exposed to the same light intensities to examine *GPX5-ARS2* induction by ROS produced endogenously. An algal strain carrying the *PSAD-ARS2* reporter gene (*Section 2.2.4* and *2.2.5*), without chemical treatment but exposed to the same light conditions, was used as a positive control of the ARS2 reaction.

After experimental treatment, colonies were removed from the agar surface and the agar media was tested for the presence of arylsulfatase enzymatic activity. To examine the activity of the enzyme on the agar, the plates were flooded with 10 mL 0.1 M Tris-HCl (EMD Chemicals) adjusted to pH 6.8 with NaOH, containing 10 mM imidazole (Sigma-Aldrich), 0.1 mg mL^{-1} α-naphthyl sulfate potassium salt (N-SO_4, chromogenic substrate; MP Biomedicals, Solon, OH, USA) and 1 mg mL^{-1} Fast Blue B Salt, and incubated at room temperature for 30 min (Ohresser *et al.* 1997). Brown/purple spots appearing on agar plates identified transformants expressing *ARS2*.

To test the induction of *GPX5-ARS2* in liquid cultures, cells were grown to a mid-log phase 3-5 x 10^6 cells mL^{-1} in TAP media, harvested by centrifugation and cell concentration was normalized to 5 x 10^6 cells mL^{-1} in TAP or TP media. Aliquots of 4.95 mL of resuspended cells were placed into each well of 12-well microtiter plates and subjected to ROS treatment by adding 50 µL of the following chemicals into separate wells: 0.5 mM NR, 0.5 mM RB, 0.5 mM DCMU (10 mM stock solution in 95% ethanol diluted with H_2O), 10 mM *t*-BOOH, and 100 mM H_2O_2 (final concentrations: 5 µM NR, 5 µM RB, 5 µM DCMU, 0.1 mM *t*-BOOH or 1 mM H_2O_2). Thus, the experiments on

liquid cultures were carried out with the addition of the same chemicals as in the case of tests performed on agar, but with lower concentrations of the NR, RB, and DCMU due to the higher availability of light and chemicals to the cells. Concentrations of the *t*-BOOH and H_2O_2 were the same as used in case of the *GPX5-ARS2* induction experiments performed on agar. Samples without the addition of the chemical were used as the controls.

The microtiter plates were incubated for 8 hours at 23°C in the dark, under ML or HL conditions. Two milliliters samples from separate wells were taken every 2 hours and centrifuged for 5 min at 2500 x g, at 23ºC. In each case, 400 µL of the supernatant was used to assess arylsulfatase activity. Reactions were initiated by adding 100 µL 0.4 M glycine buffer (pH 9.0) with 10 mM imidazole and 0.8 mM N-SO_4, followed by incubation for 1 h at 37ºC. After incubation reactions were terminated by 500 µL of 4% SDS in 0.2 M Na-acetate buffer pH 4.8 followed by the addition of 100 µL of 10 mg mL^{-1} tetrazotized-o-dianisidine (FB(B)S). Absorbance was measured immediately at 540 nm using a Beckman DU-7400 (Beckman Coulter Canada, Inc., Mississauga, ON, Canada) or a Thermo Spectronic Genesys20 model 4001/4 (Thermo Scientific, Waltham, MA, USA) spectrophotometer. The *CC-3395* strain was used as the control to zero the spectrophotometer separately for each chemical treatment, light condition and exposure time. The strain carrying the *GPX5-ARS2* without chemical treatment was used as a negative control and the strain which constitutively expressed *PSAD-ARS2* was used as a positive control.

2.3. Results

2.3.1. GPX5-ARS2 fusion allows monitoring of nuclear gene induction by light and ROS

The *C. reinhardtii* transformation approach described above produced stably transformed cell lines. Those lines maintained $^1O_2$-inducibility of *GPX5-ARS2* by HL or NR exposure for over 3 years. Figures 2.3 and 2.4 show that the *CC-3395* strain transformed with *GPX5-ARS2* displayed the expected $^1O_2$-inducibility previously reported (Leisinger *et al.* 2001). After 16 h, the cells were removed from the agar surface

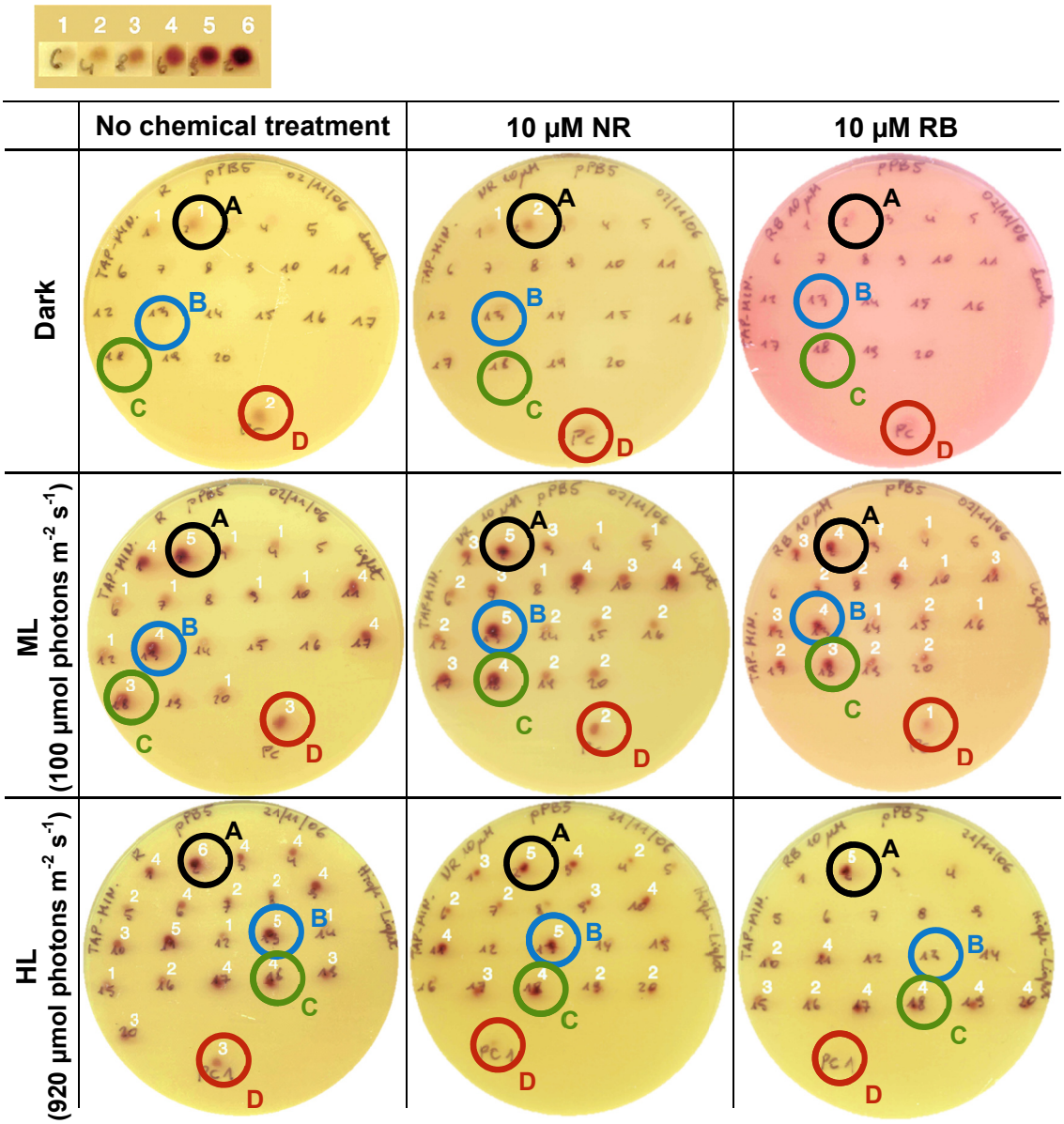

Figure 2.3. Plates of *C. reinhardtii* lines after transformation with pPB5 construct, subjected to arylsulfatase assay after treatment with 10 µM NR or 10 µM RB. Lines were tested on TP plates to induce higher photosynthetic activity. Transformants were exposed to dark, ML (100 µmol photons m^{-2} s^{-1}) or HL conditions (920 µmol photons m^{-2} s^{-1}) for 16 hours. Three lines showing the highest level of the *GPX5-ARS2* expression in response to ROS have been named *sigRep2* (A), *sigRep13* (B), and *sigRep18* (C). The line carrying *PSAD-ARS2* reporter gene (*D-sigRep*$^{+}$), being constitutively expressed, is used as the positive control (D). Scoring scale used to assess the levels of ARS2 activity on agar plates is shown on the top of the Figure.

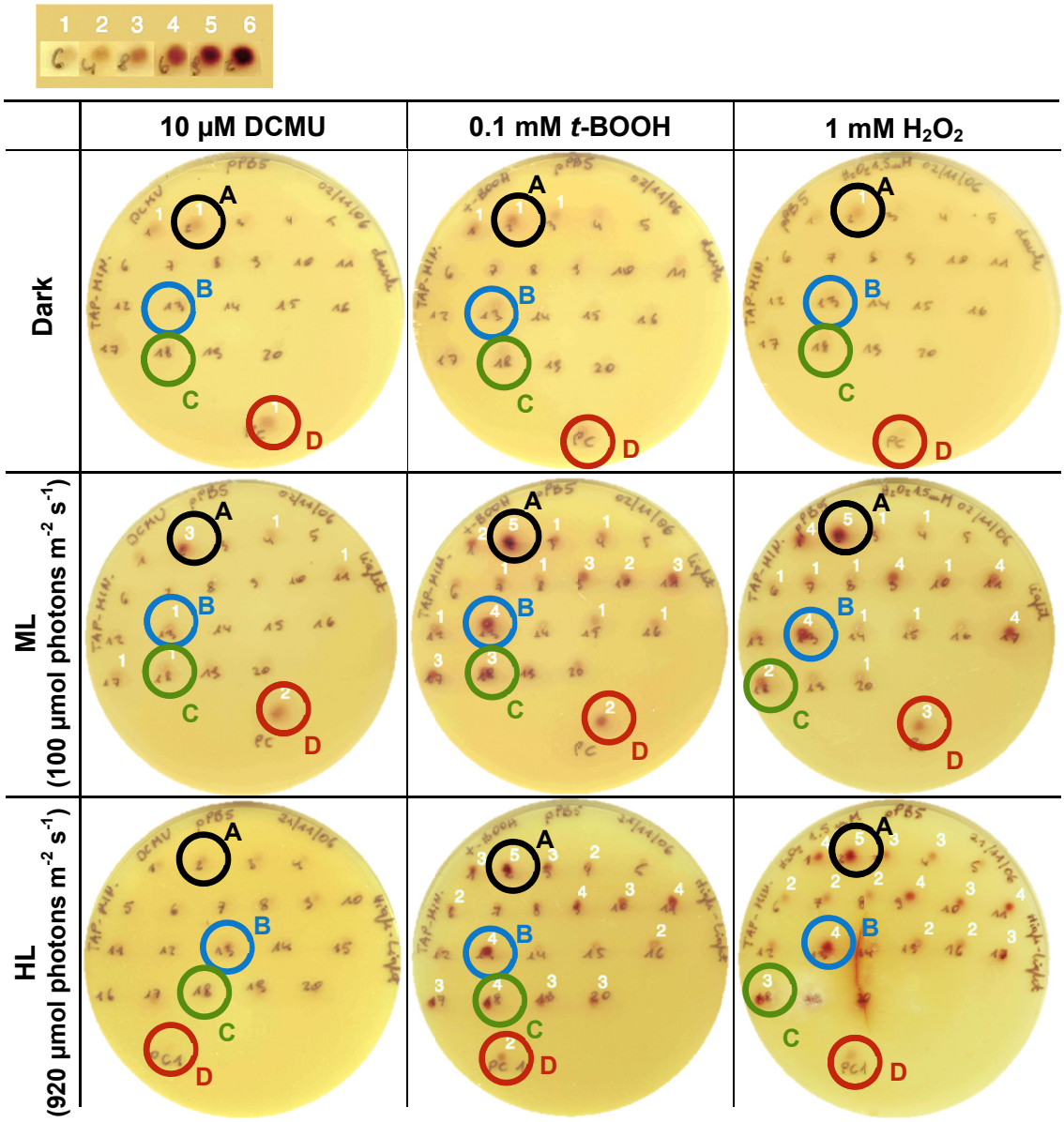

Figure 2.4. Plates of *C. reinhardtii* lines after transformation with pPB5 construct, examined for ARS2 activity after treatment with 10 µM DCMU, 0.1 mM *t*-BOOH or 1 mM H_2O_2. Lines were tested on TP plates and exposed to dark, ML or HL condition for 16 hours. The *sigRep2* (A), *sigRep13* (B), *sigRep18* (C), and *D-sigRep*$^+$ (D) are shown in circles. Scoring scale is shown on the top of the Figure.

and the plates were flooded with 10 mL of the reaction mixture described in materials and methods section (*Section 2.2.6.*). Brown-purple spots appearing on agar plates identified transformants expressing *ARS2*. Transformed colonies were scored using a scale (Figures 2.3 and 2.4) which was created based on the observed levels of ARS2 activity.

C. reinhardtii CC-3395 mutant strain transformed with pPB3 vector had 19% of colonies exhibiting arylsulfatase activity, from which one line with the highest ARS2 expression was selected and designated as *D-sigRep*$^{+}$ (*PSAD signalling reporter,* (+)-*positive control*). This line was used as a positive control on each test agar plate to ensure the ARS2-mediated reaction was proceeding as expected (Figures 2.3 and 2.4, only representative arylsulfatase activity screens are shown).

Expression of the *GPX5-ARS2* fusion in the dark was very low and difficult to observe on agar plates, regardless of the chemical treatment examined (Figure 2.3). However, exposure to either ML or HL conditions alone dramatically increased induction of *GPX5-ARS2* reporter gene (Figure 2.3). The most pronounced activity of ARS2 was observed in colonies treated with $^{1}O_2$-generating NR (Figure 2.3), *t*-BOOH, and H_2O_2 (Figure 2.4). Yet, the differences between levels of ARS2 activity and thus expression induced by these chemicals were difficult to assess on agar plates. Moderate levels of induction were observed in the case of lines treated with RB (Figure 2.3). Cells treated with DCMU when exposed to the ML conditions showed lower levels of ARS2-dependent staining when compared to ML treatment alone and no activity from the DCMU and dark or DCMU and HL treatment could be observed (Figure 2.4).

In total 35% of the colonies tested expressed *GPX5-ARS2* reporter gene in response to endogenously produced or exogenously applied ROS. Three colonies of the *C. reinhardtii CC-3395* line transformed with pPB5 were selected that consistently and reproducibly expressed high levels of ARS2 on plates in ML and HL and/or when exposed to NR but low levels of ARS2 when incubated in the dark (Figures 2.3, 2.4). Those lines were designated as *sigRep2* (*signalling reporter*), *sigRep13*, and *sigRep18* and were used for a secondary mutagenic study.

2.3.2. ARS2 activity in liquid cell cultures

Transformed colonies were also tested in liquid cell cultures in microtiter plates, to confirm previously obtained results and to semi-quantify the expression of *ARS2* in transformed lines. Figure 2.5 shows that the *sigRep2* strain displayed the expected $^1O_2$-inducibility previously reported (Leisinger *et al.* 2001). Dark expression of the *GPX5-ARS2* fusion in the liquid culture tested was very low (Figure 2.5a) while ML and NR rapidly induced the *GPX5-ARS2* reporter gene expression as measured by ARS2 activity (Figure 2.5b). On the other hand, the peroxides *t*-BOOH and H_2O_2 only caused a small induction after 8 h under ML (Figure 2.5b). HL exposure alone was enough to dramatically increase measured ARS2 activity after 8 h, i.e. 5.3-fold compared to the ML exposure (Figure 2.5, b and c) and 12.4-fold when compared to the dark conditions (Figure 2.5, a and c). Interactions between HL and exogenous ROS were reflected in the observed ARS2 activity. However, as seen in Figure 2.5c, treatment with HL and NR or DCMU led to decreased levels of ARS2 activity. On the other hand, HL and *t*-BOOH increased observed ARS2 activity after 4h, compared to HL treatment alone (Figure 2.5c).

In case of cells exposed to HL conditions, DCMU reduced the level of *GPX5* driven arylsulfatase activity (Figure 2.5c). This was especially pronounced in case of the exposure for 8 hours, where the ARS2 activity decreased to approximately 50% comparing to that observed in untreated cells. Similar results were obtained for *sigRep13* line (Figure B7.3). However, the ARS2 activity in *sigRep18* line treated with NR was higher under HL when compared to ML conditions (Figure B7.4), and expression levels observed in *sigRep2* and *sigRep13*. Results obtained from examination of the ARS2 expression in line *D-sigRep*$^+$, carrying *PSAD-ARS2* reporter gene, showed only slight increase in ARS2 activity, correlated with the exposure to higher light intensities, similar to the increase in lines *sigRep13* and *sigRep18* (Figures B7.3 and B7.4). However, ARS2 activity in *D-sigRep*$^+$ exposed to HL showed lower time-dependent increase, when compared to *sigRep2* line exposed to the same conditions (Figure B7.2).

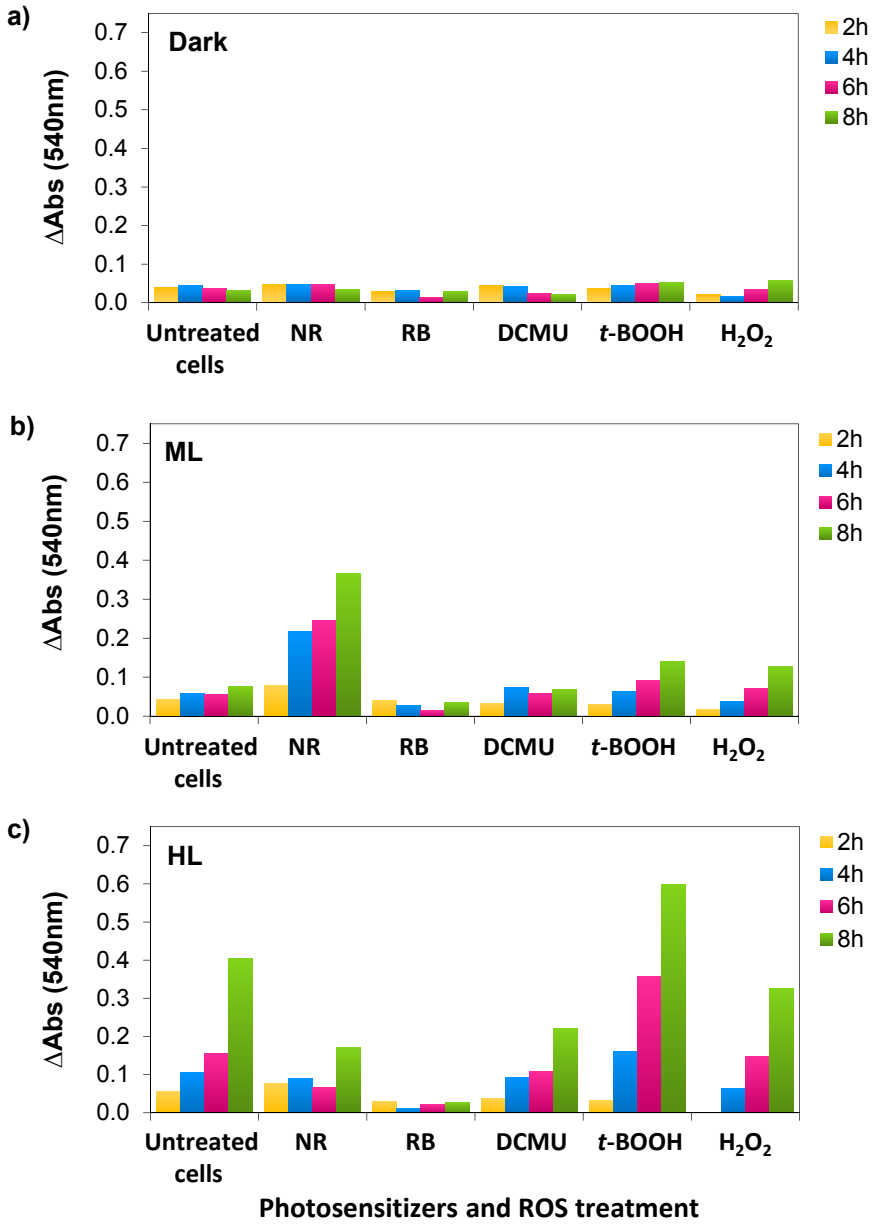

Figure 2.5. Levels of ARS2 activity in line *sigRep2*, dependent on the *GPX5*-driven *ARS2* expression, determined in liquid cell cultures exposed to oxidative stress. After addition of NR, RB, DCMU, *t*-BOOH or H_2O_2 samples were incubated at 23°C in the **a)** dark, **b)** under ML (100 µmol photons m^{-2} s^{-1}) or **c)** HL conditions (920 µmol photons m^{-2} s^{-1}). Aliquot of cells without the chemicals added was used as the control. Samples were taken every 2 h. The data shown represent averaged results obtained from an experiment performed in duplicate, raw data are shown in Figures B7.1 and B7.2.

2.4. Discussion

Although *C. reinhardtii* serves as a model organism for studying genetics or photosynthesis, the efficient tools for transformants selection are limited. This is because of the highly biased codon usage, especially in highly expressed genes (Hall *et al.* 1993) or, as it was hypothesized, extensive methylation of foreign DNA (Blankenship and Kindle 1992). Antibiotics such as paromomycin, kanamycin, or neomycin inhibit cell growth by blocking protein synthesis on ribosomes at the translocation stage (Davies and Wright 1997). Paromomycin interacts with both prokaryotic and eukaryotic ribosomes and has an especially strong effect on *C. reinhardtii* (Sizova *et al.* 2001). The aminoglycoside phosphotransferase gene (*APHVIII*) isolated from *Streptomyces rimosus* which has a codon usage and GC content close to that of nuclear DNA of *C. reinhardtii* (Danilenko *et al.* 1997) inactivates paromomycin. *APHVIII* was shown to provide an effective dominant selectable marker for *C. reinhardtii* with a transformation rate of two transformants per 1 x 10^5 cells, which is considered to be relatively high (Sizova *et al.* 2001).

C. reinhardtii cells were transformed with the pPB3 or pPB5 vectors carrying *PSAD-ARS2* or *GPX5-ARS2* reporter gene, respectively. Combining the reporter gene with a selectable marker on the same transformation vector provided an efficient transformation system and minimized potential damage to the nuclear genome, compared to co-transformation of the *ARS2* fusion plasmid along with a selectable-marker plasmid. Moreover, this approach in *C. reinhardtii* transformation also increases the chance that successfully transformed lines carry both the selectable marker and the reporter gene of interest. Therefore, this approach reduced the frequency of false positives when only the selectable marker, but not a reporter gene would be incorporated into the genome.

Fifty successfully transformed lines (30%) were randomly selected for examination of ROS-dependent *GPX5-ARS2* regulation. Transformed lines were tested for ARS2 activity on agar-solidified and in liquid TAP or TP media. Experiments performed on liquid cultures (on microtiter plates) were carried out with the addition of the same chemicals as in the case of tests performed on agar plates which allowed for a

semi-quantitative analysis of retrograde ROS signalling. Colonies that displayed ARS2 activity under ML or HL conditions were selected for further analyses.

The urea-type herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a specific photosynthesis inhibitor that binds to the second quinone acceptor (Q_B) binding site of the D1 protein of PSII, blocking electron transfer, and thus preventing reduction of the plastoquinone (PQ) pool. Previous examinations of the role of DCMU in $^1O_2$ generation in PSII, suggest that DCMU treatment leads to decreased $^1O_2$ generation, thus protecting D1 from degradation and down-regulating *GPX5* expression (Fischer *et al.* 2006; Fischer *et al.* 2007b; Flors *et al.* 2006). Based on the studies of the reaction centre in purple bacteria it was suggested that two competing charge-recombination pathways exist in PSII (Gao *et al.* 1991; Shopes and Wraight 1987). One pathway leads to the formation of radical pair $P_{680}^{\bullet+}$ and $Pheo^{\bullet-}$ (pheophytin – the first electron carrier in PSII) which can decay leading to the formation of ^{3}Chl, which in turn can react with $^3O_2$ producing $^1O_2$ (Rutherford and Krieger-Liszkay 2001). The second pathway generates $P_{680}^{\bullet+}$ and $Q_A^{\bullet-}$ without the formation of ^{3}Chl (Rutherford and Krieger-Liszkay 2001). Examination by spin trapping Electron Paramagnetic Resonance (EPR) of the ROS generation in PSII treated with different herbicides revealed that DCMU generation of $^1O_2$ is 50% lower compared to treatment with phenolic herbicides (Fufezan *et al.* 2002). This result supports the suggestion that inhibition of the PSII by the urea-type herbicide DCMU favours direct charge-recombination between $P_{680}^{\bullet+}$ and $Q_A^{\bullet-}$ which results in lower $^1O_2$ production (Fufezan *et al.* 2002; Rutherford and Krieger-Liszkay 2001). Thus, the modulating action of DCMU in fact reduces the energy available for charge-recombination after exposure to HL conditions (Fufezan *et al.* 2002; Krieger-Liszkay and Rutherford 1998; Nakajima *et al.* 1996; Rutherford and Krieger-Liszkay 2001). It was also demonstrated that DCMU does not affect the generation of $OH^{\bullet}$ or $O_2^{\bullet-}$ (Fufezan *et al.* 2002). The rationale behind application of the urea-type herbicide was to confirm the origin of the $^1O_2$ when exposing cells to HL conditions and comparing the *GPX5* induction to untreated cells. Considering its protective role in PSII this effect of DCMU was expected and it could be assumed that the reason for lower ARS2 activity was the

decrease in $^1O_2$ generation. Thus, obtained results confirmed that the major source of $^1O_2$, generated in untreated cells after exposure to HL conditions is PSII.

The partial reduction of $^3O_2$ leads to the generation of $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ (Asada 2006). The main source of H_2O_2 generation in a photosynthetic cell is PSI. Although it was demonstrated that H_2O_2 induced *GPX5* expression in *C. reinhardtii* to a lower extent than $^1O_2$ (Fischer *et al.* 2005), it has been shown to play a role as a secondary messenger regulating expression of several defence genes in higher plants, such as glutathione-s-transferases (*GSTs*), glutathione peroxidases (*GPXs*) and cytosolic ascorbate peroxidases (*APXs*; Karpinski *et al.* 1997; Karpinski *et al.* 1999; Pfannschmidt *et al.* 2001). The effect of H_2O_2 on the expression of the *GPX5-ARS2* reporter gene was examined to ensure its specificity. As expected, only a slight induction of *GPX5-ARS2* activity was observed in response to H_2O_2 when compared to other ROS treatments or HL conditions, which is also consistent with the results obtained in other studies (Fischer *et al.* 2005; Leisinger *et al.* 2001).

Organic *tert*-butyl hydroperoxide was also shown to induce *GPX5* gene expression to a minor extent when compared to $^1O_2$ under low or moderate-light conditions (Fischer *et al.* 2005). However, because of the complex nature of the ROS-mediated retrograde signalling, such as plausible cross-talk between different signalling pathways and/or possible involvement of the signal-mediating proteins in more than one signalling pathway, the role of *t*-BOOH was also examined. Although only low levels of *GPX5-ARS2* induction were observed under ML (Figure 2.5b), combination of *t*-BOOH and HL treatment caused an increased *GPX5* promoter-driven *ARS2* expression (Figure 2.5c) which is consistent with previous observations (Leisinger *et al.* 2001). As shown previously by Fischer *et al.* (2007), there are two independent signalling pathways and mechanisms inducing *GPX5* expression. The first one involves $^1O_2$ generated during HL stress while the second pathway is induced by strong prolonged photooxidative stress. It was postulated that this second pathway of protracted *GPX5* induction might be stimulated by mRNA stabilization resulting from lipid hydroperoxide formation (Fischer *et al.* 2007a). Thus, it is possible that the observed increase in *GPX5-ARS2* expression upon *t*-BOOH and HL treatment in the experiment described here was an effect of mRNA stabilization as a consequence of *t*-BOOH lipid peroxide formation. Furthermore,

lipid peroxidation by *t*-BOOH, especially in the presence of an iron catalyst (Halliwell and Gutteridge 2007), can lead to damage to the thylakoid membranes, increasing the level of photoinhibition of PSII. This would lead to increased rates of charge-recombination due to slower rates of electron flow, and hence increased production of $^1O_2$ compared to non-treated cells.

The $^1O_2$-generating photosensitizers NR and RB have been shown to up-regulate the expression of the *GPX5* gene in *Chlamydomonas reinhardtii* (Fischer *et al.* 2006; Leisinger *et al.* 2001). NR is a type I photosensitizer and a photosynthesis inhibitor (Opanasenko *et al.* 2002) while RB belongs to a group of type II photosensitizers. In both cases, the first step in reactions that involve photosensitizers is absorption of light, which results in generation of a photosensitizer in an excited state. In type I reactions, the excited photosensitizer reacts with a substrate or solvent, based on the direct H^+ or electron transfer, which leads to the formation of radicals or radical ions. In type II reactions, the excited photosensitizer reacts with triplet state oxygen ($^3O_2$) via energy transfer, which leads to the formation of $^1O_2$ (as described in more details in *Section 2.1*; Figure 2.1; Foote 1991; Marks *et al.* 1984). However, it was demonstrated using EPR spin trapping that both NR and RB stimulate $^1O_2$ generation in a light-dependent manner in isolated thylakoids (Fischer *et al.* 2004). Therefore, both NR and RB were used in this study as $^1O_2$-generating photosensitizers with expected induction of the *GPX5-ARS2* expression as an effect. Induction of the *GPX5-ARS2* by $^1O_2$ generated by NR was confirmed both on agar plates and liquid cultures examined under ML conditions. Lower levels of induction upon exposure to HL could be explained by a toxic effect of NR under this condition. Induction of *GPX5-ARS2* by $^1O_2$ produced by RB, observed under ML or HL illumination, was much lower compared to NR, both on agar plates and in liquid cultures (Figure 2.5b and c). However, subsequent experiments (presented in Figure 4.9) with the concentration of RB photosensitizer lowered to 1 μM (from initially applied 5 μM) showed even stronger induction of the *GPX5-ARS2* than 5 μM NR in the corresponding lines and light treatment (Figure 4.9). This would explain bleaching observed in all lines exposed to 10 μM RB on plates or 5 μM RB in the liquid cultures as a result of high toxicity of the RB at these concentrations, and as a consequence low expression levels of *GPX5-ARS2* (Figures 2.3 and 2.5).

The *PSAD* gene encoding subunit of photosystem I (PSI) was used to create a positive control where the *PSAD* promoter (*PSAD* 5' RR) was fused into the same *ARS2* 5' transcription start site in a single vector with paromomycin resistance cassette ($Paro^R$) used as a selectable marker (Figure 2.2). Such a system proved efficient transformation (no need for additional plasmid containing antibiotic resistance gene) and minimized potential damage to the genome. The lines transformed with the *PSAD-ARS2* reporter gene were used as a positive control to examine the utility of the *ARS2* gene fused with a foreign promoter. Hence, it was possible to compare ARS2 activity under the control of *PSAD* to ROS responsive expression driven by the *GPX5* promoter. The general trend in the induction of the ARS2 activity in cell suspension cultures of the *D-sigRep*$^+$ line, examined under the HL conditions, seemed to be lower when equated to the *GPX5*-driven activity in *sigRep2* line exposed to the same HL conditions and without chemical treatment (Figure B7.2). However, light-dependent *PSAD-ARS2* induction in *D-sigRep*$^+$ line compared to *GPX5-ARS2* induction in lines *sigRep13* (Figure B7.3) and *sigRep18* (Figure B7.4) without chemical treatment, showed the same trend under all light conditions examined.

Although the *GPX5* gene was shown previously to be specifically up-regulated by $^1O_2$ (Leisinger *et al.* 2001), recent studies have shown that the mechanisms for inducing *GPX5* gene expression could be of a much higher complexity (Fischer *et al.* 2009). It was demonstrated that GPX5 acts as a peroxidase, catalyzing the reduction of H_2O_2 and organic peroxides and that it is regulated in a thioredoxin-dependent manner, by TRXy in the chloroplasts or by TRXh1 in the cytoplasm (Fischer *et al.* 2009). The *GPX5* promoter region contains at least two independent transcription and two putatively independent translation start sites. This has the potential to produce proteins targeted either to the chloroplast or to the cytoplasm (Fischer *et al.* 2009). Four *cis*-regulatory elements located in the *GPX5* regulatory region have been identified as being required for transcriptional activation of *GPX5* by $^1O_2$, namely CRE/AP-1, TATA box, putative GC box, and localized on an antisense strand a CAAT box (Fischer *et al.* 2009; Leisinger *et al.* 1999). All those elements were shown to be required for up-regulation of *GPX5* expression by $^1O_2$ (Fischer *et al.* 2009) and all were present in the *GPX5* 5' regulatory region amplified and used in this study. The roles of two independent transcription and translation start

sites, a role in inducing expression of two versions of the *GPX5* gene and the localization of the GPX5 protein will be discussed more in Chapter 4.

In this chapter, the engineering of a *GPX5-ARS2* reporter gene, examination of the expression induced by ROS, peroxides or $^1O_2$-generating photosensitizers as well as by different light intensities were described. Also the methodology to assess activity of the ARS2 enzyme and how it correlates with the *GPX5-ARS2* expression were presented. In general, the experiments described confirmed that $^1O_2$ generated in chloroplasts is involved in retrograde signal-transduction to the nucleus (Leisinger *et al.* 2001). It was also possible to generate a *C. reinhardtii* line with an introduced engineered *GPX5-ARS2* reporter gene into the nuclear genome that allows the monitoring of changes in nuclear gene expression due to chloroplast-to-nucleus retrograde signalling based on ROS.

Although, in research presented here, the pPB5 insert copy number has not been determined in *C. reinhardtii* transformants, this aspect should be addressed in the future studies. Analyses of the transgene copy number would allow one to correlate the level of $^1O_2$-inducible *GPX5-ARS2* expression and ARS2 activity with a number of reporter gene insertions, which could have positive or negative effect on the introduced reporter gene expression but also on endogenous homologues (Hobbs *et al.* 1993).

CHAPTER 3.
SECONDARY MUTAGENESIS AND SELECTION OF THE STRAINS WITH ALTERED *GPX5-ARS2* EXPRESSION IN RESPONSE TO $^1O_2$

3.1. Introduction

Based on expression studies it has been demonstrated that *GPX5* is transcriptionally up-regulated by $^1O_2$ generated by PSII (Fischer *et al.* 2006; Fischer *et al.* 2004; Leisinger *et al.* 2001). It was also shown that *C. reinhardtii* exhibits an acclimation response to $^1O_2$, defined as the enhanced transitory tolerance triggered by endogenous or exogenously applied sub-lethal concentrations of this ROS (Ledford *et al.* 2007). These observations strongly suggest the existence of a signalling pathway that detects changes in $^1O_2$ and transmits that information from the chloroplast to the nuclear regulatory sequences. Yet, little progress has been made so far in studies attempting to explain the mechanism or characterize the constituents involved. To identify the cellular components that sense and transmit changes in ROS abundance to the nucleus, the *GPX5-ARS2* reporter strains of *C. reinhardtii,* developed and described in Chapter 2 should be an ideal system. Thus, in this chapter, a secondary mutagenic screen using the *GPX5-ARS2* reporter strains will be described. The hypothesis is that if an insertion of the transforming vector happens to disrupt the expression or function of a gene that plays a role in regulating GPX5-ARS2 expression, change in the induction of GPX5-ARS2 would be expected. This could be manifested either by abolished *GPX5-ARS2* expression when challenged by high-light stress or enhanced and constitutive expression of the reporter gene in the dark. In theory, obtained mutant strains will carry lesions in components which are responsible for controlling the expression of *GPX5* in response to $^1O_2$. Taking advantage of the positive selectable marker used for transformation of *C. reinhardtii* cells, it should be possible to identify the location of the insertion and hence the disrupted gene(s) using a PCR based approach.

3.2. Materials and methods

3.2.1. Secondary mutagenesis

To investigate the mechanism regulating *GPX5* expression in response to $^1O_2$, *C. reinhardtii* lines transformed with pPB5 (*sigRep2*, *sigRep13* and *sigRep18*) were subjected to a secondary mutagenesis by random insertion of the pSP124S plasmid which carries a bleomycin resistance cassette (Ble^R, Stevens *et al.* 1996).

Transformation of the *C. reinhardtii* cells was performed as described in *Section 2.2.5*. The pSP124s (4135 bp) plasmid was linearized with *NotI* (Fermentas) and 1 µg of DNA was used to transform 250 µL of cells at a density of 5 x 10^8 cells mL^{-1} followed by selection on TAP plates containing 15 µg mL^{-1} zeocin under low-light conditions (<10 µmol photons m^{-2} s^{-1}). Successfully transformed lines were cultured as previously described (*Section 2.2.1*) but under the low-light conditions (<10 µmol photons m^{-2} s^{-1}).

3.2.2. Selection of the strains

Lines successfully transformed with the pSL124s vector were examined for altered *GPX5-ARS2* expression in response to $^1O_2$. Zeocin resistant colonies of mutagenized *sigRep2*, *sigRep13*, and *sigRep18* were transferred to TP plates with or without 10 µM NR, followed by exposure to ML (100 µmol photons m^{-2} s^{-1}) or darkness. After 16 hours, colonies were removed from plates that were subjected to the arylsulfatase assay, as previously described (*Section 2.2.6*).

3.3. Results

GPX5-ARS2 activity of a *sigRep2*, *sigRep13* and *sigRep18* strain carrying the *GPX5-ARS2* reporter gene was demonstrated to accurately reflect the induction of the *GPX5* gene by $^1O_2$, as previously described by Leisinger *et al.* (2001). Lines *sigRep2*, *sigRep13*, and *sigRep18* were subjected to secondary mutagenesis with the pSP124s plasmid carrying the Ble^R (Stevens *et al.* 1996).

Following secondary mutagenesis, 390 colonies were obtained that were resistant to 15 µg mL^{-1} zeocin. Of these 390 colonies, 31 (~8%) displayed altered *GPX5-ARS2* reporter gene expression in response to NR-generated $^1O_2$. All of these strains were saved

while 9 were initially selected for further testing: 3 from the *sigRep2* strain, 3 from the *sigRep13* strain and 3 from the *sigRep18* strain. Results obtained from the examination of *GPX5-ARS2* expression in response to NR-generated $^1O_2$, in the lines obtained from *sigRep13* and *sigRep18* subjected to secondary mutagenesis with the pSL124s vector, are presented in Appendix C (Figures C8.1, C8.2, C8.3, C8.4, C8.5, and C8.6).

Figure 3.1 shows an image of representative TP agar plates with 40 zeocin resistant colonies of the *sigRep2* strain following secondary mutagenesis. The colonies were examined for their ability to properly regulate GPX5-ARS2 activity in response to growth in the dark or when treated with NR in the ML. As indicated by the coloured circles, a number of colonies lacked ARS2 activity in response to NR. However, no colonies were found which produced ARS2 activity in the dark. Four lines that failed to produce ARS2 activity in response to experimental conditions designated as *ΔsigRep2-4*, *ΔsigRep2-11*, *ΔsigRep2-15*, and *ΔsigRep2-16* are shown in circles A, B, C or D on the Figure 3.1, respectively.

3.4. Discussion

When this study was initiated, there were no reports of mutants altered in the expression of the *GPX5* gene. It was possible to use the reporter strains developed in Chapter 2 to screen for mutants unable to regulate GPX5, measured by the level of ARS2 activity. Thirty one zeocin resistant colonies were identified which displayed a lack of ARS2 activity when treated with NR, yet no colonies were found that displayed ARS2 activity following incubation in the dark. A recent report also produced a series of mutants in the $^1O_2$-dependent expression of *GPX5* (Fischer *et al.* 2010). Fischer *et al.* (2010) used the same *GPX5-ARS2* reporter gene as used in the study presented here. However, Fischer *et al.* (2010) used UV-mutagenesis during the secondary mutagenesis screen, to obtain 5500 colonies that showed altered transcriptional response to $^1O_2$ (Fischer *et al.* 2010). These mutant colonies were subjected to treatment with RB and NR ($^1O_2$-generating photosensitizers) or $O_2^{\bullet-}$-producing metronidazole (MZ) or methyl viologen (MV), and *t*-BOOH to characterize the *GPX5* expression profile. Fischer *et al.* (2010) identified six colonies displaying reduced expression and 32 of the examined

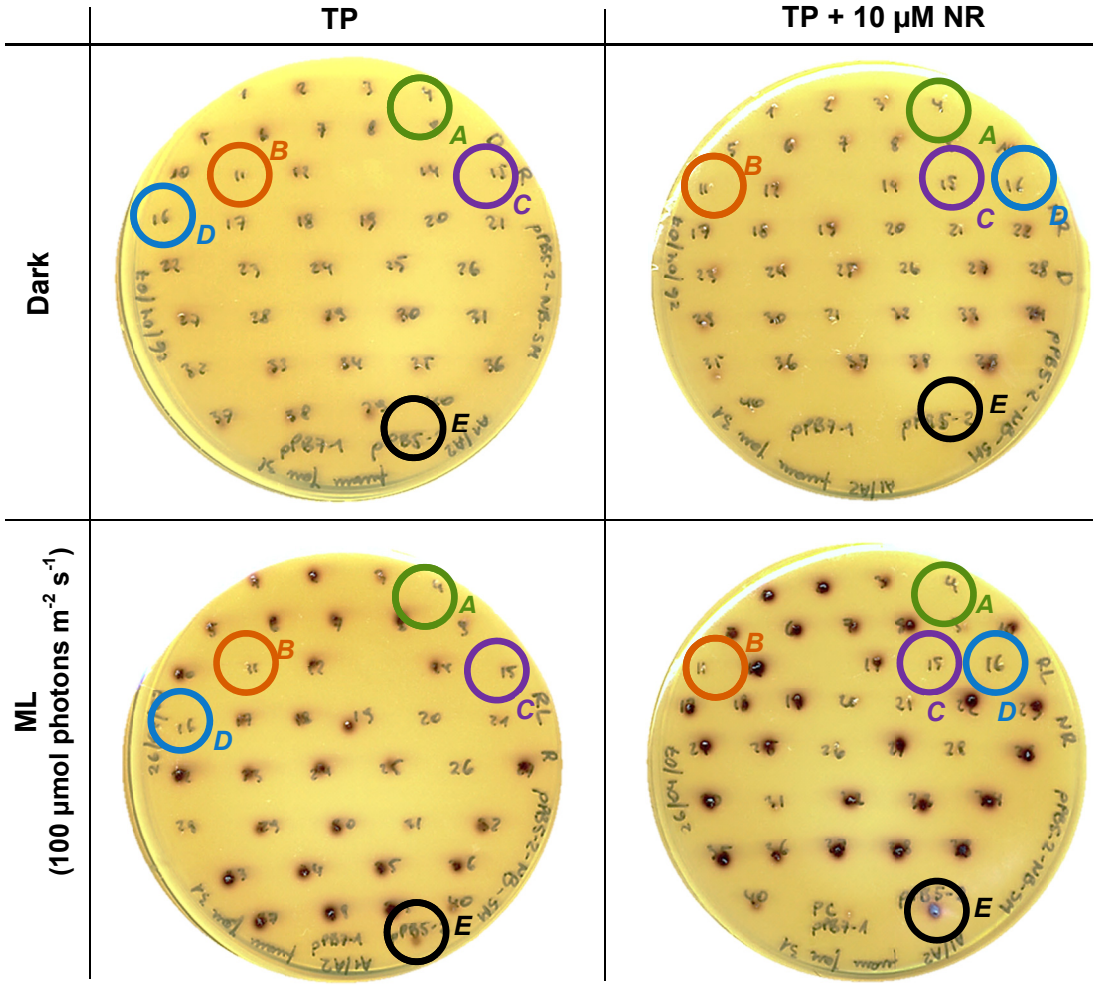

Figure 3.1. Representative samples of colonies with altered *ARS2* expression after secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep2* displaying $^{1}O_{2}$-inducible *ARS2* expression was used as a positive control (***E***). Four lines showing altered ARS2 activity in response to 10 µM NR obtained from transformation of the *sigRep2* line were designated as *ΔsigRep2-4* (***A***), *ΔsigRep2-11* (***B***), *ΔsigRep2-15* (***C***), and *ΔsigRep2-16* (***D***).

colonies exhibited increased expression of the *GPX5-ARS2* when subjected to NR treatment. A secondary screening step was performed to determine whether the level of ARS activity was reflected in the expression of the native *GPX5* gene by qRT-PCR. The expected correlation was only observed in the over-expressing strains, never in the under-expressing ones (Fischer *et al.* 2010). It was assumed that the lack of correlation between decreased ARS activity and *GPX5* transcript accumulation meant that the mutations were affecting biological steps required for the observation of ARS activity. Accordingly, the strains were not further investigated. To date the identity of the genes disrupted in the overexpressing strains have not been determined (Fischer *et al.* 2010). However, a number of the mutations appear to cause an increased accumulation of $^1O_2$, rather than a disruption in signalling per se.

In the study presented here, the secondary mutagenesis was performed using the pSP124s plasmid. This should make it much easier to identify the location of the insert and hence the mutation responsible for altered ARS2 activity. Although the possibility that the mutations may have disrupted *ARS2* expression, rather than control of the *GPX5* pathway will be also taken into account. The character of identified gene will play an important role in determining whether altered $^1O_2$-dependent regulation of *GPX5* expression is a direct or a secondary effect of the mutation. The copy number of the pSP124s plasmid insertions has not been determined but it could have an effect on the introduced Ble^R expression. Examination of the number of insertions, as mentioned in Chapter 2 (Section 2.4) can also provide valuable information about the potential damage to the nuclear genome caused by introduction of an exogenous DNA.

Although the bleomycin protein, which confers resistance to phleomycin or zeocin, does not provide a very efficient transformation system in *C. reinhardtii* (Dumas *et al.* 1994; Fuhrmann *et al.* 1999; Hayes and Wolf 1990), it was demonstrated previously to be a reliable dominant selectable marker (Ermilova *et al.* 2004; Mussgnug *et al.* 2005; Yamasaki *et al.* 2008) and was successfully used in the research described here. Thus, the transformation of all three selected *sigRep* (*sig*nalling *rep*orter) lines with the pSP124s plasmid produced zeocin resistant colonies, from which 390 in total were tested for inducibility of the *GPX5-ARS2* reporter gene by $^1O_2$. Because previous experiments determined that $^1O_2$ produced by NR can induce *GPX5-ARS2* expression, and because the

strongest ARS2 activity was observed on TP plates kept under ML conditions, the examination of the lines assayed for potentially altered signalling was limited to these experimental conditions. Additionally however, plates were kept in the dark. Such a control was necessary considering the possibility that the secondary mutagenesis could cause *GPX5-ARS2* overexpression. This could be due to a constitutively active normally $^1O_2$-stimulated signalling pathway, increased $^1O_2$ generation or decreased $^1O_2$ detoxification, as was demonstrated later by Fischer *et al.* (2010) under low-light conditions (15 µmol photons m^{-2} s^{-1}). Thus, 8% of all colonies subjected to secondary mutagenesis followed by arylsulfatase assay displayed abrogated $^1O_2$-inducible retrograde signalling. Because the variability of the chromogenic arylsulfatase assay on plates was difficult to assess precisely, colonies with complete abolishment of *GPX5-ARS2* induction were selected for further examination.

CHAPTER 4.
IDENTIFICATION OF COMPONENTS INVOLVED IN THE $^1O_2$-INDUCED CHLOROPLAST-NUCLEUS SIGNALLING PATHWAY IN *C. REINHARDTII*

4.1. Introduction

In the green alga *C. reinhardtii*, the cytosolic *GLUTATHIONE PEROXIDASE 5* (*GPX5*) is known to be up-regulated at the transcript level in response to singlet oxygen. Previous studies have shown that when the promoter region of *GPX5* is fused to the *ARYLSULFATASE 2* (*ARS2*), an effective reporter system can be generated and used to study *GPX5* expression. In Chapter 2, the construction of a single vector system for transforming *C. reinhardtii* cells with a *GPX5-ARS2* reporter gene, coupled with a positive selectable marker was described. Using this vector, stably transformed algal strains were produced, which express the ARS2 protein in a $^1O_2$-dependent manner. Three of these strains, namely *sigRep2*, *sigRep13*, and *sigRep18* were subjected to further studies. In Chapter 3, a secondary mutagenic screen using these reporter strains, and identification of a number of mutant lines that no longer regulate *GPX5-ARS2* in a $^1O_2$-dependent manner was described. The selected series of mutants were named after their parental strains as follows: *ΔsigRep2-4*, *ΔsigRep2-11*, *and ΔsigRep2-15* (Figure 3.1); *ΔsigRep13-24* (Figure C8.1), *ΔsigRep13-58* (Figure C8.2), and *ΔsigRep13-80* (Figure C8.3); and *ΔsigRep18-19* (Figure C8.4), *ΔsigRep18-84* (Figure C8.5), and *ΔsigRep18-121* (Figure C8.6).

Secondary mutagenesis was performed by random insertion of the pSP124s plasmid (Stevens *et al.* 1996) which confers resistance to the antibiotic zeocin. To identify the location within the *C. reinhardtii* genome of the pSP124s insertion a TAIL PCR-based technique will be applied. Linearizing the pSP124s (with *NotI*) prior to transformation will allow designing primers that align with the 5'-end of the insert sequence which should facilitate identification of its insertion site. Based on previous research (Dent *et al.* 2005) it is known that this type of transformation results in only about 50% of selected phenotypes being linked to the insertion of the selectable marker.

In this chapter, identification of the mutated gene responsible for the abrogated *GPX5-ARS2* $^1O_2$-inducible retrograde signalling will be described. Attempts to determine the function of the protein encoded by the identified gene, using a series of molecular and physiological experiments, will be also described. The hypothesis is that if a gene is required for the regulation of the *GPX5* promoter, its disruption will block the normal increase in the accumulation of both the native *GPX5* mRNA and the mRNA transcripts corresponding to the *GPX5-ARS2* reporter gene.

4.2. Materials and methods

4.2.1. PCR-based identification of the secondary mutants

To identify genes that were disrupted by the bleomycin resistance cassette, the Restriction-Enzyme-Site-Directed Amplification PCR (RESDA-PCR, Figure 4.1) method was used (Dent *et al.* 2005; Gonzalez-Ballester *et al.* 2005).

The selected secondary mutagenized *ΔsigRep2-15*, *ΔsigRep2-11*, and *ΔsigRep13-80* lines that displayed abolished $^1O_2$-induced signalling were grown in 250 mL Erlenmeyer flasks, on the rotary shaker (130 rpm) at 23ºC, and under low-light conditions (<10 μmol photons m^{-2} s^{-1}) to protect potentially light sensitive strains. Genomic DNA from these lines was isolated using the previously described method (as described in Chapter 2, *Section 2.2.2*).

To identify regions adjacent to an inserted DNA marker (Ble^R) degenerate primers Deg-*TaqI*, Deg-*AluI*, Deg-*SacII*, and Deg-*PstI* (Gonzalez-Ballester *et al.* 2005) were used (Figure 4.1, Table A6.1) that partially anneal with respective sequences of *TaqI*, *AluI*, *SacII* or *PstI* restriction sites in combination with primers KW8 and PB16 which are specific for the bleomycin resistance cassette (Figure 4.1, Table A6.1). All PCRs were carried out in the iCycler thermal cycler (Bio-Rad) with *Taq* DNA Polymerase (recombinant, Fermentas) accordingly to manufacturer's instructions, with 10X *Taq* buffer with KCl, dNTP Mix (0.2 mM each; Fermentas), $MgCl_2$ adjusted to 3 mM, and 4% (v/v) DMSO.

The first round of RESDA-PCR was carried out using the KW8 primer with the set of degenerate primers (Deg-*TaqI*, Deg-*AluI*, Deg-*SacII*, and Deg-*PstI*) in four separate

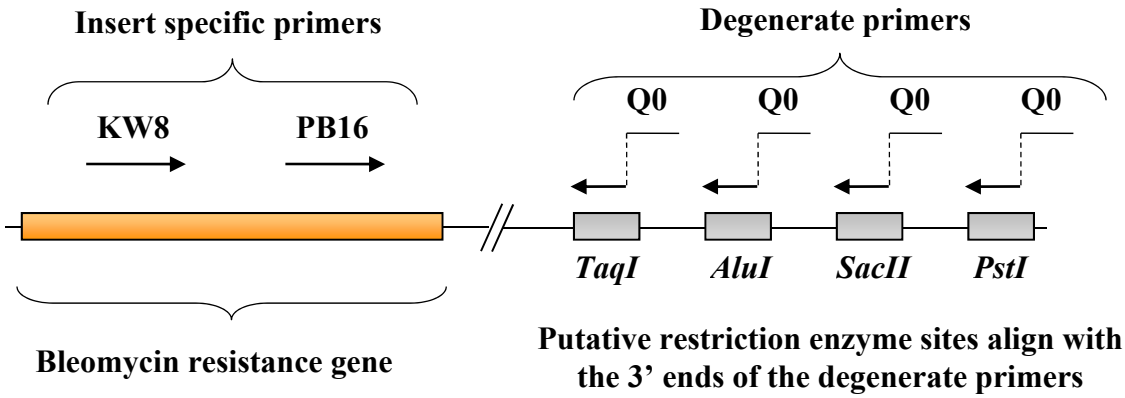

Figure 4.1. Restriction Enzyme Site Directed PCR (RESDA-PCR, modified from González-Ballester *et al.* 2005). Primer KW8 specific for the bleomycin resistance cassette was used together with the degenerate primers Deg-*TaqI*, Deg-*AluI*, Deg-*SacII*, and Deg-*PstI*. The Q0 primer and PB16 (second bleomycin resistance cassette specific primer) were used to identify RESDA-PCR product.

reactions, following the protocol depicted in Table 4.1a. Amplicons in amount of 1 µL from the 1st round of PCR was used as templates for the 2nd round reactions using primers PB16 and Q0 (Table 4.1b).

The amplified DNA fragments were cloned into pJET1/blunt Cloning Vector (GeneJET PCR Cloning Kit, Fermentas) and sequenced (NRC–PBI). Sequences were compared to the *C. reinhardtii* genome sequence using Blastn (DOE Joint Genome Institute, http://genome.jgi-psf.org/chlamy/chlamy.home.html).

4.2.2. RNA isolation

Total RNA was isolated using the modified phenol-chloroform method as described previously by Ledford *et al.* (2007). To isolate RNA from experimental lines, cells were collected by centrifugation (2500 x g, 3 min) and resuspended in 0.5 mL H_2O followed by addition of 0.5 mL 2X Lysis Buffer (600 mM NaCl; J.T. Baker, Phillipsburg, NJ, USA), 10 mM EDTA (EMD Chemicals), 100 mM Tris-HCl (EMD Chemicals), and 4% (w/v) SDS (BioShop) and 10 µL of Proteinase K stock solution (40 mg mL^{-1}; Omega Bio-Tek). After incubation at 65ºC (10-15 min), 75 µL of 4 M KCl (EMD Chemicals) was added and samples were kept on ice for 15 min, followed by centrifugation at 21,250 x g for 10 min at 4ºC. After centrifugation samples were extracted twice with 0.75 mL of phenol:chloroform (1:1, EMD Chemicals) followed by 0.75 mL chloroform (EMD Chemicals) extraction to remove trace amounts of phenol.

In the first approach 0.33 volumes 8 M LiCl (EMD Chemicals) was added to the sample and samples were kept at 4ºC overnight to precipitate RNA followed by centrifugation at 21,250 x g for 15 min at 4ºC. Pellets were washed with 500 µL 70% ethanol, allowed to dry on ice and resuspended in 30-50 µL of TE buffer (10 mM Tris, 0.1 mM Na_2EDTA, pH 8.0). In the second approach, instead of overnight precipitation with 8 M LiCl, the RNA was purified using a HIBind RNA mini column (E.Z.N.A. Plant RNA kit, Omega Bio-Tek) according to the manufacturer's instructions.

Alternatively, following experimental treatment, the cells were harvested by centrifugation, snap-frozen using LN_2, and the total RNA was isolated by the acid guanidine isothiocyanate-phenol-chloroform method using TRIzol Reagent (Invitrogen) according to manufacturer's protocol. In all cases the quantity and purity of RNA was

Table 4.1 Reaction conditions for **a)** 1st round (Gonzalez-Ballester *et al.* 2005; Liu and Whittier 1995), modified) and **b)** 2nd round RESDA-PCR.

a)

RESDA-PCR, 1st round reaction			
cycle	**step**	**°C**	**min**
1(1X)	1	96	5:00
2(5X)	1	95	1:00
	2	60	1:00
	3	72	2:00
3(1X)	1	95	1:00
	2	25	3:00
ramping to 72 °C, over			3:00
	3	72	2:00
4(20X)	1	95	1:00
	2	60	1:00
	3	72	2:00
	4	95	1:00
	5	60	1:00
	6	72	2:00
	7	95	1:00
	8	40	1:00
	9	72	2:00
5(1X)	1	72	10:00
6(1X)	1	4	∞

b)

RESDA-PCR, 2nd round reaction			
cycle	**step**	**°C**	**min**
1(1x)	1	96	5:00
2(35X)	1	95	1:00
	2	60	1:00
	3	72	1:00
3(1X)	1	72	10:00
4(1X)	1	4	∞

assessed at 260 nm calculating the A_{260}/A_{280} ratio using a Beckman DU-7400 (Beckman Coulter Canada, Inc., Mississauga, ON, Canada) or Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA) spectrophotometer, and compared to the optimal of ~2.0. The quality of the extracted RNA was examined by 1% (w/v) agarose (EMD Chemicals) non-denaturing gel electrophoresis (Sambrook and Russel 2001). All the RNA samples were stored at -80ºC.

4.2.3. sqRT-PCR analysis of the PSBP2 transcript levels in the ΔsigRep2-11 mutant line and 5'RACE

Transcript levels were examined for the *PSBP2* gene in the *ΔsigRep2-11* mutant strain (*psbP2*) and in the *CC-3395* line (positive control) using semi-quantitative RT-PCR method (sqRT-PCR). Transcript profiles were determined for cells grown under GL (50 μmol photons m^{-2} s^{-1}) or treated with 0.5 μM RB (Sigma-Aldrich) and returned to GL conditions, for 1 h, at 23ºC.

Total RNA was isolated and quantified based on spectrophotometer quantifications (OD_{260}) and 1 μg of total RNA was used for cDNA synthesis after treatment with DNaseI (2.5 μL / 5μg RNA; Fermentas) for 30 min at 37ºC, followed by inactivation by addition of 1 μL 25 mM EDTA (Fermentas) and incubation at 65ºC for 10 min. To generate cDNA the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) was used according to the manufacturer's instruction. RNA samples were treated with RiboLock RNase Inhibitor (protects mRNA degradation in temperatures up to 55ºC, Fermentas) and the reverse transcriptase reaction was performed using RevertAid H Minus Reverse Transcriptase and a oligo$(dT)_{18}$ primer included in the kit, in an iCycler thermal cycler (Bio-Rad) according to the cycling protocol: 37ºC for 10 min, 45ºC for 70 min, and 70ºC for 10 min.

The *18S* rRNA was used as a loading control after amplification with primers PB18S-F and PB18S-R (Table A6.2), using DreamTaq Polymerase (Fermentas), 10X DreamTaq Buffer, and dNTP Mix (0.2 mM each; Fermentas), following the protocol: initial denaturation at 95ºC for 3 min, 35 cycles of 95ºC for 30 s, 56ºC for 30 s, and 72ºC for 30 s and a final extension at 72ºC for 10 min. The DNA products obtained from

amplification of the *18S* rRNA were visualized on EtBr-stained 5% (w/v) agarose gel and the size of the amplicons was assessed using Ultra Low Range DNA Ladder (SM 1213, Fermentas).

Transcript levels of the *PSBP2* gene were examined using KW212 and KW215 in PCR performed on the cDNA templates from *CC-3395* and *ΔsigRep2-11*. The PCR with KW212 and KW215 primers was performed using DreamTaq Polymerase (Fermentas), 10X DreamTaq Buffer, dNTP Mix (0.2 mM each; Fermentas), with addition of DMSO (Fermentas, 4% (v/v) final concentration) as follows: initial denaturation at 95ºC for 3 min, 35 cycles of 95ºC for 30 s, 63ºC for 30 s, and 72ºC for 1 min and a final extension at 72ºC for 10 min. The DNA products from the amplification of the *PSBP2* transcripts were visualized by EtBr-stained 1% (w/v) agarose gel electrophoresis, as described previously (*Section 2.2.3*), and the size of amplified fragments was assessed using GeneRuler 1 kb DNA Ladder (SM 0313, Fermentas).

5'-RACE (Rapid Amplification of cDNA Ends) was performed on the *CC-3395* and *psbP2* strains using a commercially available kit (5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Invitrogen), following the manufacturer's instructions. The synthesis of first strand cDNA was performed using 0.5 µg of RNA as the template. The SuperScript II RNase H Minus reverse transcriptase provided in the kit was used with an antisense gene specific primer KW198 (Table A6.2) in a reaction performed at 42ºC for 1 hour. The product of the reaction was purified and dC-tailed using the Terminal deoxynucleotidyl transferase (TdT). The 5'-RACE-PCR was conducted with recombinant *Taq* DNA polymerase (Fermentas) using a polyG- anchor primer and a gene specific primer KW173 (Table A6.2). The reactions were carried out using the iCycler thermal cycler (Bio-Rad). Nested PCR products were purified from an agarose gel, then blunt-ligated into the pJet1.2/blunt vector provided in the CloneJet PCR Cloning Kit (Fermentas), cloned into *E. coli* and sequenced (NRC–PBI).

4.2.4. Analysis of the effect of the insertion on PSBP2 neighbouring genes

The insertional mutagenesis of the *C. reinhardtii* genome was previously shown to cause deletions from a few bases to 60 kb or more in the DNA surrounding the insertion as a result of genomic DNA rearrangement associated with exogenous DNA

integration (Matsuo *et al.* 2008; Page *et al.* 2004; Tam and Lefebvre 1993). Thus, to examine how much if any surrounding genomic DNA was deleted during the insertion event, PCR was performed on genomic DNA from the *ΔsigRep2-11* line and also *CC-3395* and *sigRep2* as the controls. DNA was obtained using the Phire Plant Direct PCR Kit (Finnzymes OY, Espoo, Finland) following the "Dilution protocol", as described by the manufacturer. Although this kit was originally designed for direct PCR on fresh or frozen plant material (such as leaves or seeds), it proved to also be suitable for direct PCR on algae strains (Blair Skrupski, personal communication). Using a toothpick, a minute amount of algae was picked from each colony on the plate and placed in 20 µL Dilution Buffer provided in the kit and incubated at room temperature for 3 min. Cell debris was pelleted and 0.5 µL of supernatant was used directly in the PCR mixture.

To determine the size of the deletions, a genomic DNA region (locus Cre16.g678850) approximately 3000 bp upstream of the insertion site was tested. Primers KW209 and KW210 (Table A6.2) were used in the PCR using Phire Hot Start DNA polymerase and 2X Phire Plant PCR Buffer (includes dNTPs and $MgCl_2$) provided in the kit, and following manufacturer's instructions. All the reactions were performed in an iCycler thermal cycler (Bio-Rad) with initial denaturation at 98ºC for 10 min, followed by 40 cycles of 98ºC for 5 s, 65ºC for 5 s, and 72ºC for 20 s, and a final extension at 72ºC for 1 min.

To determine if a second neighbouring gene, predicted to be a sensor histidine kinase-related was still present in the *ΔsigRep2-11* line (locus Cre16.g678900), PCR was performed on genomic DNA using the same Phire^R Plant Direct PCR Kit and methodology with KW207 and KW208 primers (Table A6.2). PCR conditions were the same as in the case with the KW209 and KW210 primers, except the annealing temperature that was raised to 68ºC.

Also, to ensure that the gene at the Cre16.g678900 locus was still transcribed, the presence of its mRNA was examined by diagnostic RT-PCR using the KW207 and KW208 primers (Table A6.2) in the *ΔsigRep2-11*, *sigRep2*, and *CC-3395* lines. Algae were grown under continuous cool white-light illumination (GL, 50 µmol photons m^{-2} s^{-1}) in 100 mL TAP in 250 mL Erlenmeyer flasks, on the rotary shaker (130 rpm), at 23ºC until reaching the mid-log phase of 3-5 x 10^6 cells mL^{-1}. Total RNA was isolated as

previously described (*Section 4.2.2*) and reverse transcriptase reactions were performed using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas). cDNA obtained from *ΔsigRep2-11*, *sigRep2*, and *CC-3395* lines were normalized based on the amplicons obtained for the *CBLP* gene encoding a cytosolic G-protein β-subunit-like protein (Schloss 1990). Amplification of *CBLP* was carried with KW50 and KW51 primers (Table A6.2) using DreamTaq Polymerase (Fermentas), 10X DreamTaq Buffer, dNTP Mix (0.2 mM each; Fermentas), and following the protocol: initial denaturation at 95ºC for 3 min, 35 cycles of 95ºC for 30 s, 63ºC for 30 s, and 72ºC for 1 min and a final extension at 72ºC for 10 min. Thus, the cDNA amounts for all lines were adjusted according to the levels of DNA amplicons obtained from these reactions.

The final PCRs with KW207 and KW208 primers were carried out with DreamTaq DNA Polymerase (Fermentas), 10X DreamTaq Buffer, dNTP Mix (0.2 mM each; Fermentas), with the addition of 10% (v/v) DMSO (Fermentas) and the following cycling conditions: initial denaturation at 95ºC for 3 min, 35 cycles of 95ºC for 30 s, 63ºC for 30 s, and 72ºC for 1 min, followed by a final extension at 72ºC for 10 min. Products of the reactions were examined by 1% (w/v) agarose gel electrophoresis, as previously described (*Section 2.2.3*; Sambrook and Russel 2001).

4.2.5. Cloning the wild-type PSBP2 gene

To confirm that the psbP2 protein was responsible for the abrogated $^1O_2$-dependent *GPX5-ARS2* activity in the *ΔsigRep2-11* strain, the attempt was undertaken to rescue the ROS signalling phenotype by transforming the cells with a genomic copy of the *PSBP2* gene. Genomic DNA from the *CC-3395* strain was isolated as described in *Section 2.2.2*, and nested PCR was performed using PB33 and PB34 primers for the first round of amplification and PB35 and PB36 for the second round of PCR (Table A6.2). The PCRs were carried out in an iCycler thermal cycler (Bio-Rad) using Long PCR Enzyme Mix (Fermentas) according to manufacturer's instructions, with 10X Long PCR Buffer (without $MgCl_2$), dNTP Mix (0.2 mM each; Fermentas), with the addition of 8% (v/v) DMSO and an $MgCl_2$ concentration adjusted to 0.75 mM. Reaction conditions were the same for the first round with primers PB33 and PB34 as for the second round with primers PB35 and PB36 (Table A6.2). The following PCR protocol was used: initial

denaturation at 95ºC for 4 min, 35 cycles of 94ºC for 45 s, 60ºC for 45 s, and 72ºC for 3 min 30 s and a final extension at 72ºC for 10 min.

To obtain the cDNA of the *PSBP2* mRNA transcript, RNA from the *CC-3395* strain was isolated as previously described (*Section 4.2.2*) followed by RT-PCR using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) using the oligo(dT)$_{18}$ primer as per the manufacturer's instructions. The cDNA template was amplified by PCR using primers PB28 and PB29 (Table A6.2). PCR conditions conducted with primers PB28 and PB29 were the same as with PB33 and PB34 or PB35 and PB36.

Both genomic and cDNA copies of *PSBP2* were cloned into the pTZ57R/T vector (InsTAclone PCR Cloning Kit, Fermentas), following manufacturer's instructions, used to transform *E. coli* following the previously described transformation method (Chapter 2, *Section 2.2.3*), and sequence verified.

4.2.6. Rescue of the ROS signalling phenotype

Taking advantage of the arginine auxotrophy phenotype (*arg7*) of the *CC-3395* strain used for the primary mutagenesis, the genomic copy of *PSBP2* was cloned into an *ARG7* containing plasmid (T7-Arg7; Auchincloss *et al.* 1999) obtained from the Chlamydomonas Center. To do this, *PSBP2* was cut from the pTZ57R/T vector using *KpnI* and *SalI* endonucleases and ligated (using T4 DNA Ligase, Fermentas, according to manufacturer's instructions) into the corresponding sites of the T7-Arg7 plasmid, to yield the pP2-T7Arg7 vector (Figure 4.2a). The pP2-T7Arg7 was verified by sequencing (NRC–PBI).

In an attempt to rescue the *psbP2* phenotype, the pP2-T7Arg7 vector was linearized with *KpnI* and introduced into the genome of the *psbP2* strain cells by electroporation (as described in *Section 2.2.5*) and the transformants were selected on plates of arginine deficient TAP media.

Rescue was also attempted by cloning the genomic *PSBP2* gene into the pHyg3 plasmid (obtained from Dr. Wolfgang Mages, Universität Regensburg, Germany) which contains a hygromycin B resistance cassette (Berthold *et al.* 2002). In this case the *PSBP2* gene was cut from the pTZ57R/T vector using *NruI* (*Bsp68I*) and *StuI* (*Eco 147I*)

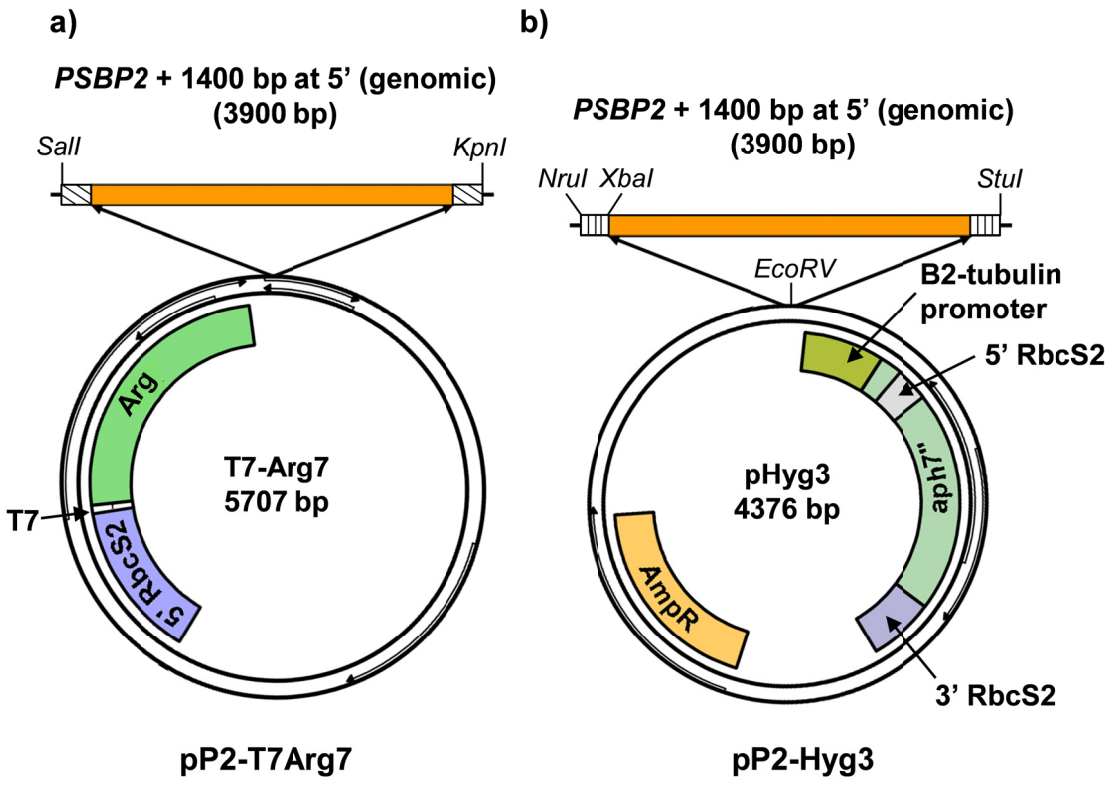

Figure 4.2. Plasmids carrying a genomic version of the *PSBP2* gene used for the independent transformation of the *C. reinhardtii psbP2* mutant line. **a)** The T7-Arg7 plasmid containing arginine cassette with *PSBP2* insert generated pP2-T7Arg7 plasmid. Sequences resulting from cutting *PSBP2* from the pTZ57R/T vector with *SalI* and *KpnI* are indicated as boxes with a diagonal pattern, the same *KpnI* site was used to linearize the pP2-T7Arg7. **b)** The pHyg3 plasmid carrying hygromycin B resistance cassette after insertion of the *PSBP2* generated pP2-Hyg3 vector. In this case sequences resulting from cutting *PSBP2* from the pTZ57R/T vector with *NruI* and *StuI* are indicated as boxes with a vertical pattern. The *EcoRV* insert site and *XbaI* used to linearize pP2-Hyg3 for *psbP2* line transformation are indicated.

and blunt-end ligated using T4 ligase (Fermentas, accordingly to manufacturer's instruction) into the *EcoRV* site of the pHyg3 plasmid. The resulting plasmid was designated as pP2-Hyg3 (Figure 4.2b) and its DNA sequence was verified by sequencing (NRC–PBI). One microgram of the *XbaI* (Invitrogen) linearized pP2-Hyg3 was used for the electroporation of the *psbP2* line, followed with selection performed on TAP plates supplemented with 10 μg L^{-1} hygromycin B (Invitrogen, San Diego, CA, USA).

Successfully transformed colonies from two independent transformations with pP2-T7Arg7 (Figure 4.2a) or pP2-Hyg3 (Figure 4.2b) plasmid were assayed on plates, as described previously (*Section 2.2.6*), for singlet oxygen dependent ARS2 activity to determine whether the *PSBP2* genomic fragment could restore $^1O_2$-inducible *GPX5-ARS2* expression.

To confirm the potentially rescued $^1O_2$-inducible signalling phenotype in lines transformed with pP2-T7Arg7 or pP2-Hyg3 plasmids, lines obtained from these transformations were also examined for $^1O_2$-inducible *GPX5-ARS2* expression in liquid cultures (as described in *Section 2.2.6*). Only one experiment was performed to test three lines obtained from transformation with pP2-T7Arg7, while lines obtained from transformation with pP2-Hyg3 were tested in three independent experiments with three replicates per trial (n = 9). Statistical analyses were performed using analysis of variance (ANOVA) in Minitab 15 software (Minitab Inc., State College, PA, USA); means were separated by using 95% confidence intervals based on pooled standard deviation.

4.2.7. Growth response of the psbP2 mutant to different light intensities

Growth characteristics of the *psbP2* line were compared to the following: *CC-3395*, *sigRep2*, and previously characterized *CC-4142* (de Vitry *et al.* 1989; Mayfield *et al.* 1987; Rochaix 1987). Line *CC-4142 (Fud* 39 mt^+*)* carries a *psbP1* mutation (synonymous to the *oee2*, *oxygen evolving enhancer protein 2*); it was obtained from the Chlamydomonas Center (University of Minnesota, St. Paul, MN). All lines were grown in TAP under GL (50 μmol photons m^{-2} s^{-1}) conditions until reaching a mid-log phase of 3-5 x 10^6 cells mL^{-1}. Samples of each culture were harvested by centrifugation (1000 x g) and resuspended in TAP or TP media, normalizing the cell concentration to 5 x 10^7 cells mL^{-1}. Aliquots of 10 μL of each sample were placed on TAP or TP plates and placed in

the dark, under GL or HL conditions for 4 days. Relative growth of colonies was then assessed by differences in overall appearance.

4.2.8. Photosynthetic characterization of psbP2

To ensure that the *psbP2* mutation did not alter photosynthesis and decrease $^1O_2$-dependent *GPX5* expression as a result of altered metabolism, the *in vivo* photosynthetic capability and the ability of the mutant cells to respond to photoinhibitory conditions was examined using pulse-amplitude modulated chlorophyll fluorescence. Cells were grown in TP media at 23°C and continuous cool white-light illumination of 50 μmol photons m^{-2} s^{-1}, until they reached mid-log phase. To assess the photosynthetic capability of the *psbP2* mutant cells in comparison to the *sigRep2* strain, light-response curves were produced. Cells were placed in a temperature-regulated cuvette and dark adapted for 10 min. Using a XE-PAM system (Heinz Walz GmbH, Effeltrich, Germany), the chlorophyll fluorescence signal was collected using the attached PDA -100 (Heinz Walz GmbH). Minimal fluorescence (F_o) was elicited using the Xe-modulated light source. To determine maximal dark-adapted fluorescence (F_m) or steady-state maximal fluorescence (F_m') an 800 ms pulse of 4000 μmol photons m^{-2} s^{-1} was applied. Steady-state fluorescence measurements involved exposing the cells to increasing light intensities. Once steady-state fluorescence was reached (F_s), a saturating pulse was applied, the steady-state light was extinguished and a low-level far-red light source was turned on (102-FR Heinz Walz GmbH), allowing the steady-state minimal fluorescence (F_o') to be measured.

The photosynthetic parameter qL was calculated using the equation $qL = [(F_m' - F_s)/(F_m' - F_o')] \times (F_o'/F_s)$ (Kramer *et al.* 2004), and converted to 1-qL to estimate the reduced level of PSII under the steady-state conditions being examined. The non-photochemical quenching parameter NPQ was calculated as $NPQ = (F_m-F_m')/F_m'$ (Genty *et al.* 1990). The NPQ estimates the amount of energy being dissipated by PSII and its antenna rather than being used for photochemistry.

To examine whether the PSBP2 protein was involved in protecting PSII from high-light-mediated damage or aiding in the repair of damaged PSII following photoinhibition, cells were exposed to 1200 μmol photons m^{-2} s^{-1} at 22 °C, in the

presence or absence of 50 μg mL^{-1} chloramphenicol for 90 minutes, followed by recovery under 20 μmol photons m^{-2} s^{-1} for 60 min. To calculate $F_v/F_m = F_m-F_o/F_m$ during these measurements, samples were removed from the exposed culture and dark adapted for 10 min, before determining F_o and F_m.

4.2.9. Functional complementation of PSBP1 and PSBP2

To determine whether the PSBP1 protein can rescue the mutant $^1O_2$-inducible signalling phenotype in selected *psbP2* mutants or whether the PSBP2 protein can complement the function of the psbP1 protein in the *CC-4142* (*oee2-psbP1*) mutant line, a cross-transformation with wild-type genes encoding these proteins was conducted. Furthermore, a control experiment aimed to rescue the *psbP1* mutation in the *CC-4142* mutant line by introducing the wild-type gene encoding PSBP1 protein was performed.

For transformation experiments involving the *PSBP2* gene, the previously constructed pP2-Hyg3 plasmid was used (*Section 4.2.6*). In an attempt to rescue the *psbP1* mutation in the *CC-4142* mutant line or to examine if $^1O_2$-signalling in *psbP2* can be rescued with the PSBP1 protein, a wild-type *PSBP1* gene was amplified using genomic DNA isolated from *CC-3395* as previously described in Chapter 2 (*Section 2.2.2*). PCRs were conducted in an iCycler thermal cycler (Bio-Rad) using Phusion High-Fidelity DNA Polymerase (generates blunt-ended products, New England Biolabs, Inc., Pickering, ON, Canada) with 5X Phusion HF Buffer and dNTP Mix (0.2 mM each; Fermentas), according to the manufacturer's recommendations. Reactions were performed with PB41 and PB42 primers (Table A6.2), in a 2-step PCR following the protocol: initial denaturation at 98ºC for 30 s, 30 cycles of 98ºC for 10 s and 72ºC for 1 min 45 s with a final extension at 72ºC for 10 min. This allowed the amplification of a 3437 bp fragment which was blunt-end-ligated into the pJet1.2/blunt vector provided in the CloneJet PCR Cloning Kit (Fermentas) used to transform in *E. coli* (Sambrook and Russel 2001) and sequenced (NRC–PBI). The *PSBP1* gene was cut out from the pJet1.2/blunt vector with *EcoRV* (Fermentas) and blunt-end-ligated into the pre-digested *EcoRV* site of pHyg3 (Berthold *et al.* 2002) using T4 ligase (Fermentas) to give the pP1-Hyg3 plasmid which was subsequently used to transform *E. coli* (Sambrook and Russel 2001). Subsequent pP1-Hyg3 plasmid was verified by control digestions with *EcoRV* and

HindIII (Fermentas) and used to transform the *CC-4142* (*psbP1*) and *psbP2* lines by electroporation as previously described (Chapter 2, *Section 2.2.5*). Following transformation of the *psbP2* and *CC-4142* mutant lines with the pP1-pHyg3 vector or the *CC-4142* line with the pP2-Hyg3 plasmid, successful transformants were selected on TAP plates supplemented with 10 μM hygromycin B under low-light conditions (≤ 10 μmol photons m^{-2} s^{-1}).

Lines obtained from transformation of *psbP2* (mutant line with an abrogated $^1O_2$-inducible signalling phenotype) with the pP1-Hyg3 plasmid were tested on plates by examining the *GPX5-ARS2* response to NR-generated $^1O_2$ or HL conditions, as described in *Section 4.2.6*. Lines obtained from the transformation of the *CC-4142* mutant strain with pP1-pHyg3 or pP2-pHyg3 plasmid were tested for the ability to survive HL exposure on TAP or TP plates for 4 days, as described in *Section 4.2.7*.

4.2.10. Acclimation to singlet oxygen in psbP2 mutant line

The *CC-3395*, *sigRep2*, and *psbP2* lines were grown in liquid TAP, supplemented with 2.5 mg L^{-1} arginine, on the rotary shaker (130 rpm), under continuous cool white-light illumination of 50 μmol photons m^{-2} s^{-1} (GL). After reaching the mid-log phase (3-5 x 10^6 cell mL^{-1}) cells were harvested by centrifugation (1500 x g for 3 min) and resuspended in TAP, normalizing cell concentration for all lines to 5 x 10^6 cells mL^{-1}. Aliquots of 200 mL of cells of each line were divided into two equal volumes and placed in two 250 mL Erlenmeyer flasks. To pre-adapt cells to higher $^1O_2$ conditions, RB was added (2 μM final concentration) to one of the two flasks containing *CC-3395*, *sigRep2*, or *psbP2* cultures. The other set of cultures was used as the control. All lines for RB pre-treatment and controls were grown on the rotary shaker (130 rpm), under GL, at 23ºC. After 1 hour cells were washed to remove the RB by centrifugation (1500 x g for 3 min) and resuspended in 100 mL TAP media. Suspension of 10 mL control or RB pre-treated *CC-3395*, *sigRep2*, and *psbP2* cultures was pipetted into six 15 mL falcon tubes and challenged with different RB concentrations as follows: 0, 4, 8, 12, 16, and 20 μM for 1 hour on the rotary shaker (130 rpm) and under the same GL and temperature conditions. After the oxidative challenge, cells were harvested by centrifugation (1500 x g for 3 min) and resuspended in 10 mL TAP to remove residual RB. Aliquots of 10 μL resuspended

cells were pipetted onto TAP plates and growth was examined following incubation under GL, at 23ºC, after 9 days.

The same experimental procedure was repeated to test singlet oxygen acclimation under more challenging conditions on TP plates. Thus instead of in TAP media, cells were pre-treated with 2 μM RB, washed, $^1O_2$-challenged, washed again and put on plates containing TP (without reduced carbon source). Similarly, all lines were kept under the same conditions and examined after 9 days.

4.2.11. Examination of GPX5 and GPX5-ARS2 transcript abundance

To address the recent finding that a long and short version of the GPX5 transcript could be produced, the relative accumulation of both mRNA transcript versions of the *GPX5* gene (Fischer *et al.* 2009), and corresponding short and long versions of the *GPX5-ARS2* fusion were examined. The transcript profiles for *GPX5* and the *GPX5-ARS2* reporter gene were examined in the *CC-3395* (positive control for *GPX5* and a negative control for the *GPX5-ARS2*), the *sigRep2*, the *psbP2*, and one of the lines which displayed rescued $^1O_2$-inducible signalling phenotype, *RΔsig-P2H-4*. Lines were exposed to GL (50 μmol photons m^{-2} s^{-1}) or treated with 0.5 μM RB and returned to GL conditions.

Total RNA from all lines was isolated by TRIzol method, as described in *Section 4.2.2*, and the RT reaction performed as described in *Section 4.2.3*. The resulting cDNAs from all samples were examined according to *18S* amplification with primers PB18S-F and PB18S-R (Table A6.2; as described in *Section 4.2.3*), and the amplicons were used as a loading control.

For the short version of the *GPX5* transcript, primers KW235 and KW236 were used, and for the long version, primers KW233 and KW234 (Table A6.2; Fischer *et al.* 2009). These primers allow one to distinguish between the transcript levels of the endogenous *GPX5* in all lines examined. All the PCRs on synthesized cDNA templates were performed using an iCycler thermal cycler (Bio-Rad) and DreamTaq DNA Polymerase (Fermentas) with 10X DreamTaq Buffer, dNTP Mix (0.2 mM each; Fermentas); or with DreamTaq PCR Master Mix (2X, Fermentas), according to manufacturer's instruction. The reaction conditions for KW235 and KW236 were as

follows: initial denaturation at 95ºC for 3 min, 35 cycles of 95ºC for 30 s, 54ºC for 30 s, and 72ºC for 30 s and a final extension at 72ºC for 10 min. PCRs with KW233 and KW234 were performed according to the protocol: initial denaturation at 95ºC for 3 min, 26 cycles of 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 30 s with a final extension at 72ºC for 10 min. Reactions were optimized for each primer pair with regard to annealing temperatures.

To examine transcript levels of the introduced *GPX5-ARS2* reporter gene, primer pairs KW 237 (aligning with the *ARS2* gene) and KW230 (amplifying the short version of the *GPX5* promoter) or KW233 (amplifying the long version of the *GPX5* promoter) were used (Table A6.2), to produce amplicons corresponding to the short or long versions of the exogenous *GPX5-ARS2*. The reaction conditions for short (primers KW230 and KW 237) and for the long version of the *GPX5-ARS2* (primers KW233 and KW237) were the same: initial denaturation at 95ºC for 3 min, 26 cycles of 95ºC for 30 s, 56ºC for 30 s, and 72ºC for 30 s with a final extension at 72ºC for 10 min.

Amplification of the *18S* transcript was performed as previously described (*Section 4.2.3*). All amplicons of *18S*, short and long *GPX* and *GPX5-ARS2* were examined by electrophoresis on a 5% (w/v) agarose gel stained with EtBr, as described previously. The sizes of amplified DNA were assessed using GeneRuler Ultra Low Range DNA Ladder, ready to use, 10-300 bp (SM1213, Fermentas).

4.3. Results

4.3.1. PCR-based analysis allowed identification of the mutated gene

In an attempt to identify the gene(s) affected in the colonies identified in Chapter 3 which showed an altered *GPX5-ARS2* mediated ARS2 activity, RESDA-PCR was performed on total genomic DNA isolated from selected lines with altered $^1O_2$-inducible signalling: *ΔsigRep2-15*, *ΔsigRep2-11*, and *ΔsigRep13-80*. However, only in the case of the *ΔsigRep2-11* insertional mutant did the RESDA-PCR yield a DNA fragment suitable for further analysis. That PCR product was successfully cloned and sequenced (NRC–PBI). Subsequent bioinformatic analysis (based on the *Chlamydomonas* genomic resources) showed that the fragment aligned with a sequence on chromosome 16,

corresponding to bases 4299614 to 4299700 (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). This placed the pSP124s insertion in the first exon of an annotated gene which had previously been designated as *PSBP2* due to the presence of a conserved *PSBP* domain (Pfam: 01789; www.phytozome.net).

Figure 4.3 depicts the annotated *PSBP2* gene based on the genomic DNA sequence obtained from the publicly available portal of JGI (DOE Joint Genome Institute, Walnut Creek, CA, www.phytozome.net, locus Cre16.g678800) and mapped using Vector NTI 11 software (Invitrogen) and pDRAW32 (AcaClone, www.acaclone.com). As can be seen in Figure 4.3 representing ~2.4 kb *PSBP2* gene, the border of the plasmid insertion is in the 5' UTR region, 5 bases upstream of the predicted ATG start codon (Figure 4.3). However, the insertion is in reverse orientation to the *PSBP2* sequence and therefore 87 bp of the 3'-end fragment of the *PSBP2* 5'UTR adjacent to the Ble^R insert was identified (Figure 4.3). Because the mutation has been identified, the *ΔsigRep2-11* was renamed *psbP2* and subjected to further studies.

4.3.2. The psbP2 mutant produces reduced levels and a truncated version of the PSBP2 transcript, as revealed by sqRT-PCR and 5'RACE, respectively

Comparative analysis of *PSBP2* transcript levels was conducted on total mRNA isolated from *CC-3395* and *psbP2* mutant lines, after subjecting algae to experimental conditions, as described in *Section 4.2.3*. As can be seen in Figure 4.4, the amount of PCR amplicons produced in the sqRT-PCR is lower in the *psbP2* than *CC-3395*. This was true both between the lines and in the case of different experimental treatments. Sequencing of the amplicons ensured that the correct gene was assayed. Map of the *PSBP2* mRNA indicating primer pairs KW212 and KW215 are indicated in Figure 4.4. The nucleotide sequence with primers alignment is provided in Figure D9.1.

5' RACE was applied to analyze synthesized *psbP2* mRNA in the mutant line, because the insertion was in the 5' UTR. The reaction was performed on mRNA isolated from *CC-3395* and *psbP2* following exposure to GL conditions. Comparative analysis of mRNA isolated from *CC-3395* and *psbP2* indicated that only a truncated form of the *psbP2* transcript was present in the mutant line with abrogated $^1O_2$-inducible signalling pathway. As can be seen in Figure 4.5, 324 bp, 274, and 89 bp 5'RACE fragments from

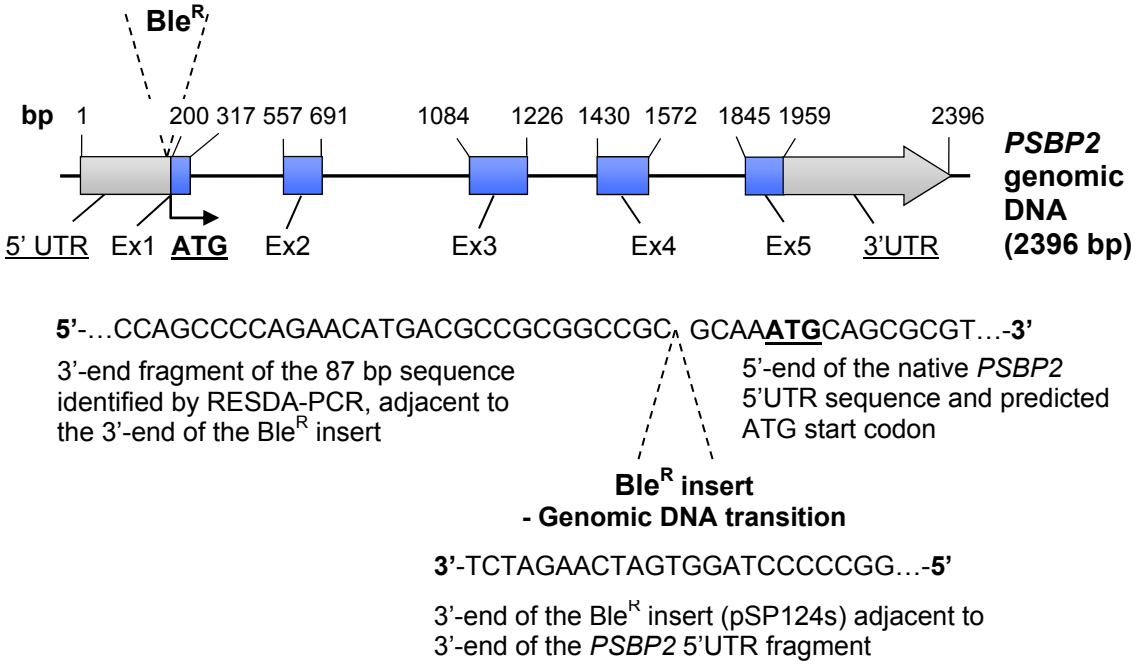

Figure 4.3. A genomic map of the *PSBP2* gene in *C. reinhardtii*. The location of the pSP124s (Ble^R) insertion (in reverse orientation) to the 5' UTR region of the *PSBP2* gene is marked by the dashed arrowhead; 5' and 3' UTRs indicated in grey and the 5 exons indicated in blue.. The model was constructed based on genomic DNA sequence obtained from JGI (locus Cre16.g678800; DOE Joint Genome Institute, www.phytozome.net) and analyzed using Vector NTI 11 and pDRAW32 software.

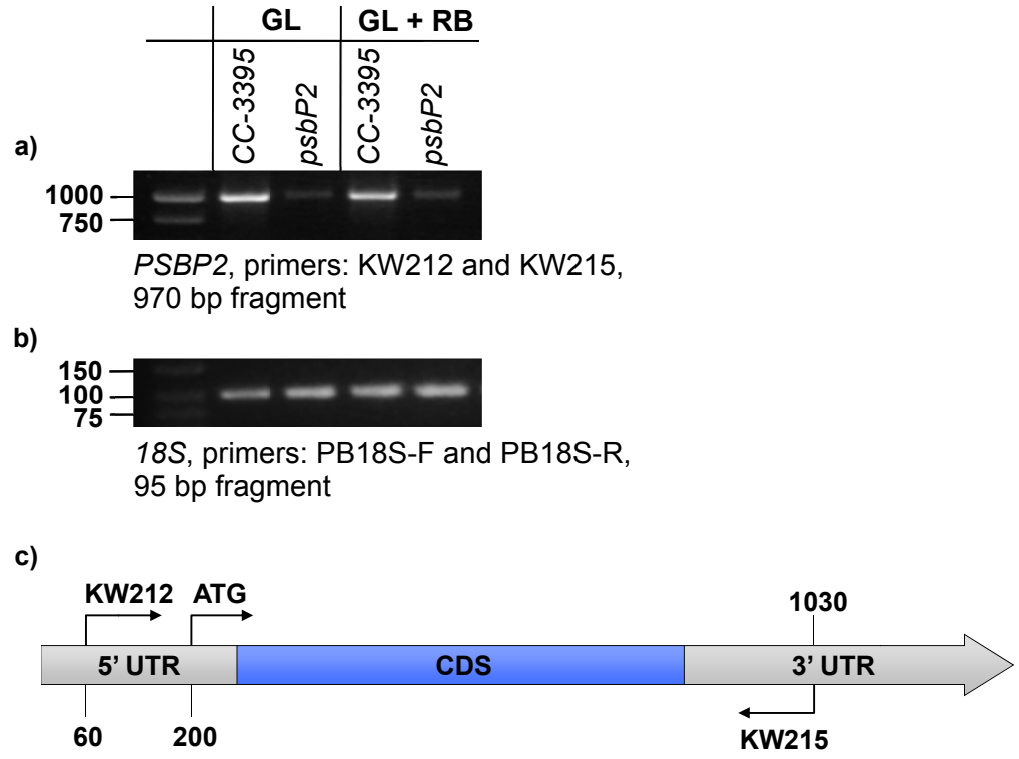

Figure 4.4. A semi-quantitative RT-PCR analysis of the transcript levels of the *PSBP2* gene. Lines were kept for 1 h in the dark, under GL conditions (50 µmol photons m^{-2} s^{-1}) or under GL conditions with the addition of 0.5 µM RB. Transcript abundance was examined in the *CC-3395*, and *psbP2*. **a)** The *PSBP2* transcript levels were determined using primers KW212 and KW 215. **b)** The 18S amplification with primers PB18S-F and PB18S-R was used as a loading control. **c)** Map of the *PSBP2* transcript with indicated primer pairs KW212/KW215 and predicted ATG start codon. *PSBP2* mRNA sequence with indicated pairs of primers used for sqRT-PCR analysis can be found in Figure D9.1.

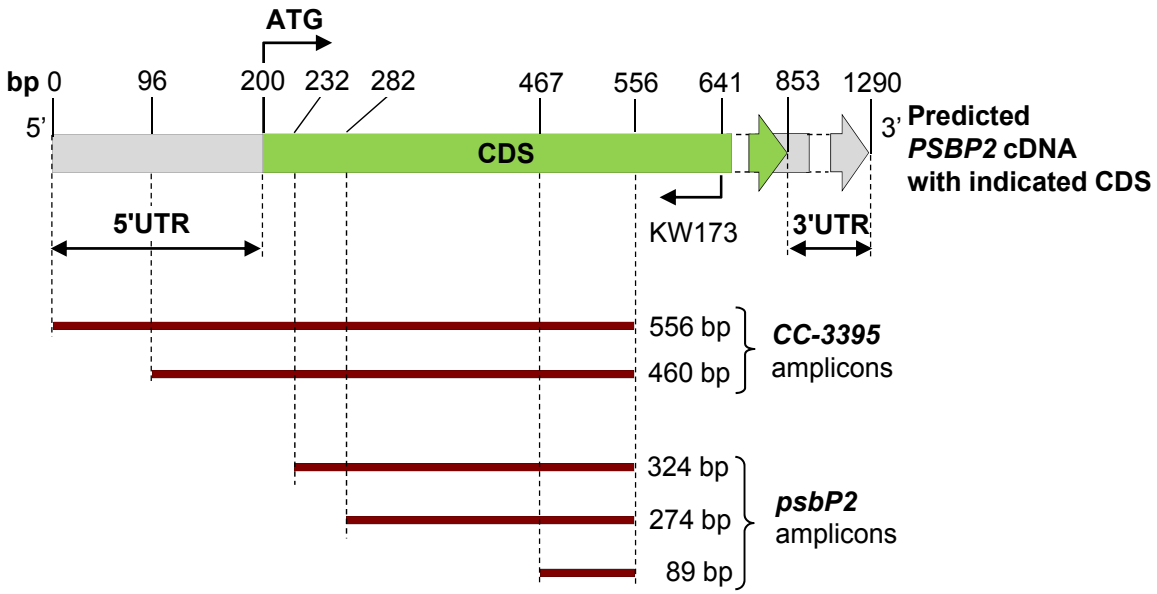

Figure 4.5. A schematic representation of the results obtained from 5'RACE on *CC-3395* and *psbP2*, indicating the predicted *PSBP2* cDNA, coding sequence and sequenced amplicons. The gene-specific primer KW198 was used for the cDNA generation (not shown) and a nested, gene-specific KW173 for amplification of the tailed cDNA. Figure was constructed based on data provided by Dr. Ken Wilson.

the *psbP2* strain were missing the 5'UTR, the predicted ATG start codon, and between 32 to 267 bases of coding sequence (Figure 4.5). Thus, it can be assumed that no functional *PSBP2* protein is produced.

4.3.3. The pSP124S insertion did not affect PSBP2 neighbouring genes

In previous studies large genomic DNA fragments (up to 65 kb) were reported to be deleted during insertional mutagenesis in *C. reinhardtii* (Matsuo *et al.* 2008; Tam and Lefebvre 1993). Hence, it was determined how much, if any, of the surrounding *psbP2* genomic DNA was deleted during the insertion event.

Figure 4.6a was constructed based on the sequence data available on the JGI database (www.phytozome.net) and analysis was performed using the Vector NTI 11 program. It shows a fragment of *C. reinhardtii* chromosome 16 with the *PSBP2* gene, and the corresponding site of the Ble^R insertion (Figure 4.6a). Two putative genes were identified upstream of the *PSBP2* gene regions: one encoding a putative hydroxymethylglutaryl-CoA synthase (locus Cre16.g678850) and the second one identified as containing a putative response-regulator-receiver domain (sensor histidine kinase-related, locus Cre16.g678900; Figure 4.6a).

To examine if these genes had been affected by the Ble^R insertion, PCR on genomic DNA was performed using primers KW209 and KW210, which indicated that the region approximately 3000 bp upstream of the insertion site was still present and intact (locus Cre16.g678850, Figure 4.6b). To ensure a neighbouring gene predicted to be a two-component signalling component was present and expressed normally (locus Cre16.g678900, Figure 4.6a), PCR was performed on genomic DNA using primers KW207 and KW208 (Figure 4.6c). Because this gene is more likely to encode signalling component than a putative hydroxymethylglutaryl-CoA synthase, diagnostic RT-PCR using the same set of primers KW207 and KW208 (Figure 4.6d) was performed to determine if it was still transcribed. As shown in Figure 4.6d, in each of the *CC-3395*, *sigRep2*, and *psbP2* lines this putative signalling gene was expressed normally in cells grown under GL conditions.

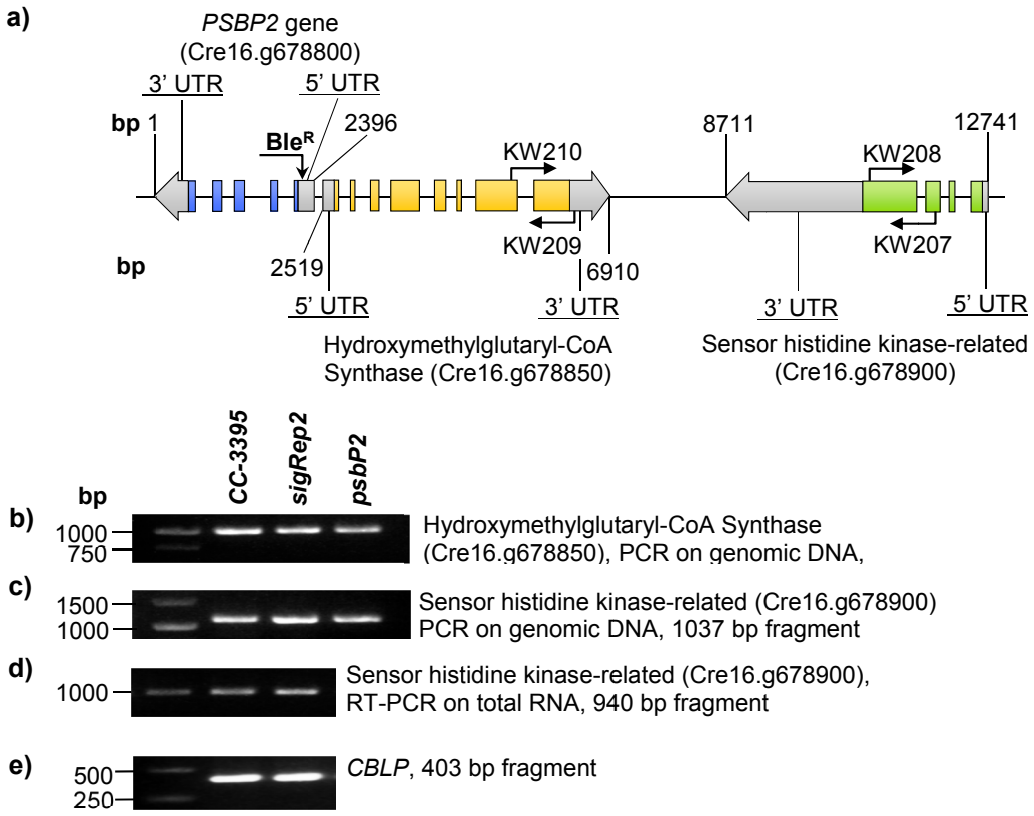

Figure 4.6. Section of *C. reinhardtii* chromosome 16 and examination of the *PSBP2* neighbouring genes by PCR and RT-PCR. **a)** Location of the *PSBP2* gene (Cre16.g678800), a putative hydroxymethylglutaryl-CoA synthase (Cre16.g678850), and a sensor histidine kinase-related gene (Cre16.g678900). The 5' and 3' UTRs are represented by the grey boxes and exons as the coloured boxes. Binding sites of the primers KW209, KW210, KW207, and KW208 used for the examination of the genomic DNA sequences or transcript are indicated. **b)** PCR on genomic DNA testing the regions upstream of the insertion site using primers KW209 and KW210 (locus Cre16.g678850) and **c)** primers KW207 and KW208 (locus Cre16.g678900). **d)** RT-PCR examination of the Cre16.g678900 transcript (primers KW207 and KW208). **e)** The cDNA was normalized based on the sqRT-PCR amplification of the *CBLP* gene fragment using KW50 and KW51 primers. The model was constructed based on genomic DNA sequence obtained from JGI (DOE Joint Genome Institute, www.phytozome.net) and analyzed using Vector NTI 11 software.

4.3.4. Analysis of the cloned PSBP2 gene

Based on the predicted gene model (Figure 4.3), oligonucleotides were designed to PCR-amplify cDNA and genomic versions of *PSBP2*. The genomic version of the *PSBP2* gene that was amplified extends approximately 2400 bp from the transcriptional start site to the end of the 3' untranslated region. The predicted gene model was confirmed by the sequencing of the cDNA for the *PSBP2* gene that was obtained by RT-PCR, and predicts a protein of 23 kDa with a putative N-terminal transit peptide for chloroplast localization (Figure 4.7). Based on bioinformatics analysis using the InterProScan (Zdobnov and Apweiler 2001), the 217 amino acid *C. reinhardtii* PSBP2 protein contains a MOG1/PSBP domain between amino acids 59 and 186, and a twin-arginine translocation motif in the first 40 amino acids (Figure 4.7). This is consistent with results of the TatP analysis program (Bendtsen *et al.* 2005) which suggested a signalling peptide with a cleavage site between amino acid residues 38 and 39, leaving a 19 kDa mature protein (Figure 4.7).

The WoLF PSORT program makes localization predictions using a *k*-nearest neighbour algorithm, adjusted to possible dual localization, and based on amino acid sequence, composition, sorting signals, and functional motifs (Horton *et al.* 2007; Horton *et al.* 2006). Analysis of the 217 amino acids sequence by the signal peptide prediction method using the WoLF PSORT (http://wolfpsort.org/), indicated with high probability score the PSBP2 protein of *C. reinhardtii* to be chloroplast-localized (certainty factor of 11), and with low probability score to be mitochondria-localized (with a score of 3; Horton et al. 2007; Horton et al. 2006).

4.3.5. Rescue of the $^1O_2$-induced signalling in the psbP2 line

Lines obtained by transformation of the *psbP2* mutant with pP2-T7Arg7 or pP2-Hyg3 plasmid were tested for $^1O_2$-inducible *GPX5-ARS2* expression on plates and in liquid cultures by the semi-quantitative method for assessing ARS2 activity by absorbance measurements. Figure 4.8 shows representative results from 8 lines obtained from transformation of the *psbP2* line with the pP2-T7Arg7 plasmid. Lines were tested on TP plates in the dark, exposed to ML or HL conditions, in combination with or without 10 μM NR. As shown in Figure 4.8, approximately 40% of the lines exhibited

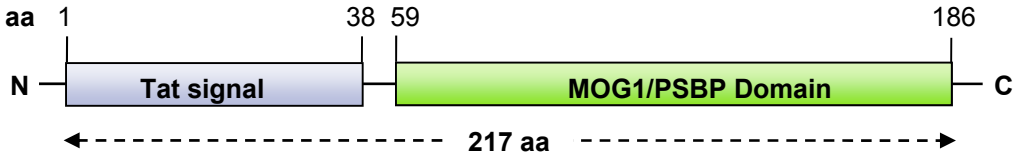

Figure 4.7. The PSBP2 protein model of *C. reinhardtii*, indicating the Twin-arginine translocation pathway (Tat pathway signal peptide) motif and the MOG1/PSBP domain.

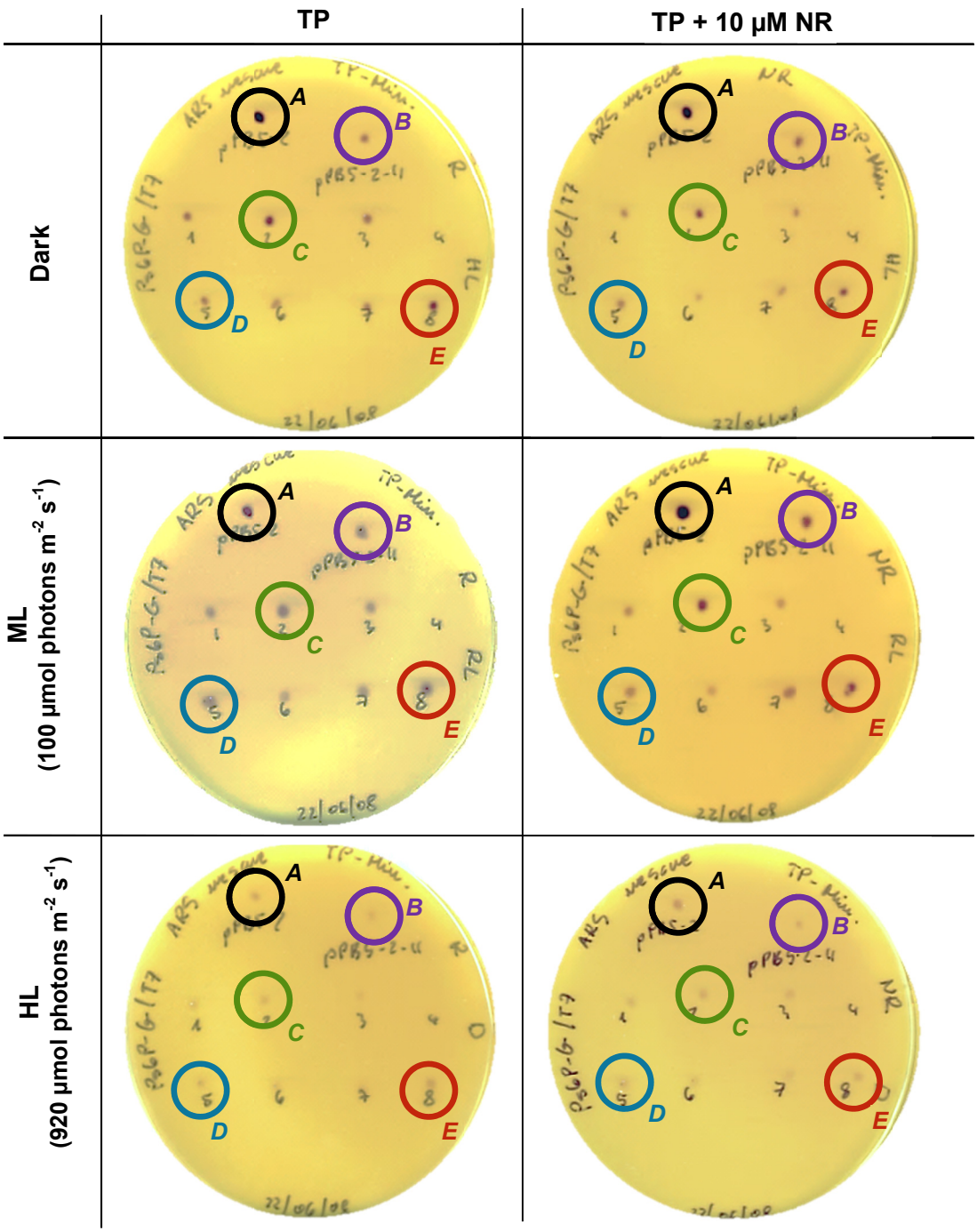

Figure 4.8. Colonies transformed with pP2-T7Arg7 plasmid tested on TP plates with or without NR. Lines: *sigRep2* (***A***), *psbP2* (***B***), and lines with recovered ARS2 activity: *RΔsig-P2A-2* (***C***), *RΔsig-P2A-5* (***D***), and *RΔsig-P2A-8* (***E***) are marked in circles.

$^1O_2$-dependent induction of *GPX5-ARS2* activity that was especially pronounced in the case of TP with 10 µM NR and ML. These colonies have been named *RΔsig-P2A-2* (rescued delta signalling with *PSBP2* in T7-Arg7, Figure 4.8C), *RΔsig-P2A-5* (Figure 4.8D), and *RΔsig-P2A-8* (Figure 4.8E), and were subjected to examination of the ARS2 activity of TP liquid cell cultures in response to 5 µM NR, 0.1 mM *t*-BOOH or 1 mM H_2O_2 (Figure 4.9). The *sigRep2* line (Figure 4.8A) was used as a positive control while the *psbP2* mutant line (Figure 4.8B) acted as the negative control for all treatments.

As can be seen on the Figure 4.9, three lines (*RΔsig-P2A-2*, *RΔsig-P2A-5*, and *RΔsig-P2A-8*) displayed ARS2 activity when treated with NR and exposed to ML conditions, while activity following dark treatment was very low in all lines. Treatment with NR and exposure to HL conditions proved to be lethal, most likely because of the high $^1O_2$ levels being produced. Interestingly, treatment with *t*-BOOH and HL caused elevated levels of ARS2 activity. H_2O_2 and HL seemed to have only a slightly positive effect on *GPX5-ARS2* expression as observed in *RΔsig-P2A-2*, *RΔsig-P2A-5*, and *RΔsig-P2A-8* when compared to *sigRep2* but still higher than *psbP2*.

Lines transformed with the pP2-Hyg3 plasmid were also examined for *GPX5-ARS2* activity in response to ROS. Figure 4.10 illustrates a representative example of lines tested on TP plates with or without 10 µM NR. As in the case of lines transformed with the pP2-T7Arg7 plasmid, samples were kept under dark conditions or exposed to ML or HL. Also in this case, approximately 40% of transformants tested (24 can be seen on plates) displayed a rescued $^1O_2$-dependent inducible signalling phenotype when exposed to ML conditions or when treated with NR, while transformation with an empty vector control did not restore ARS2 activity (Figure 4.11). Lines *CC-3395* (Figure 4.10A) and *psbP2* (Figure 4.10B) were used as negative controls while *sigRep2* (Figure 4.10C) as a positive control. Three colonies were selected and named *RΔsig-P2H-4* (rescued delta signalling with *PSBP2* in pHyg3, Figure 4.10D), *RΔsig-P2H-17* (Figure 4.10E), and *RΔsig-P2H-20* (Figure 4.10F). These lines were subjected to further examination of $^1O_2$-dependent *GPX5-ARS2* activity in liquid cultures (Figure 4.12). Figure 4.12 depicts graphs showing higher ARS2 activity measured in lines rescued with the pP2-Hyg3 plasmid treated with NR and exposed to ML conditions when compared to the *psbP2* line. Also higher levels of ARS2 activity were observed in samples exposed to high-light.

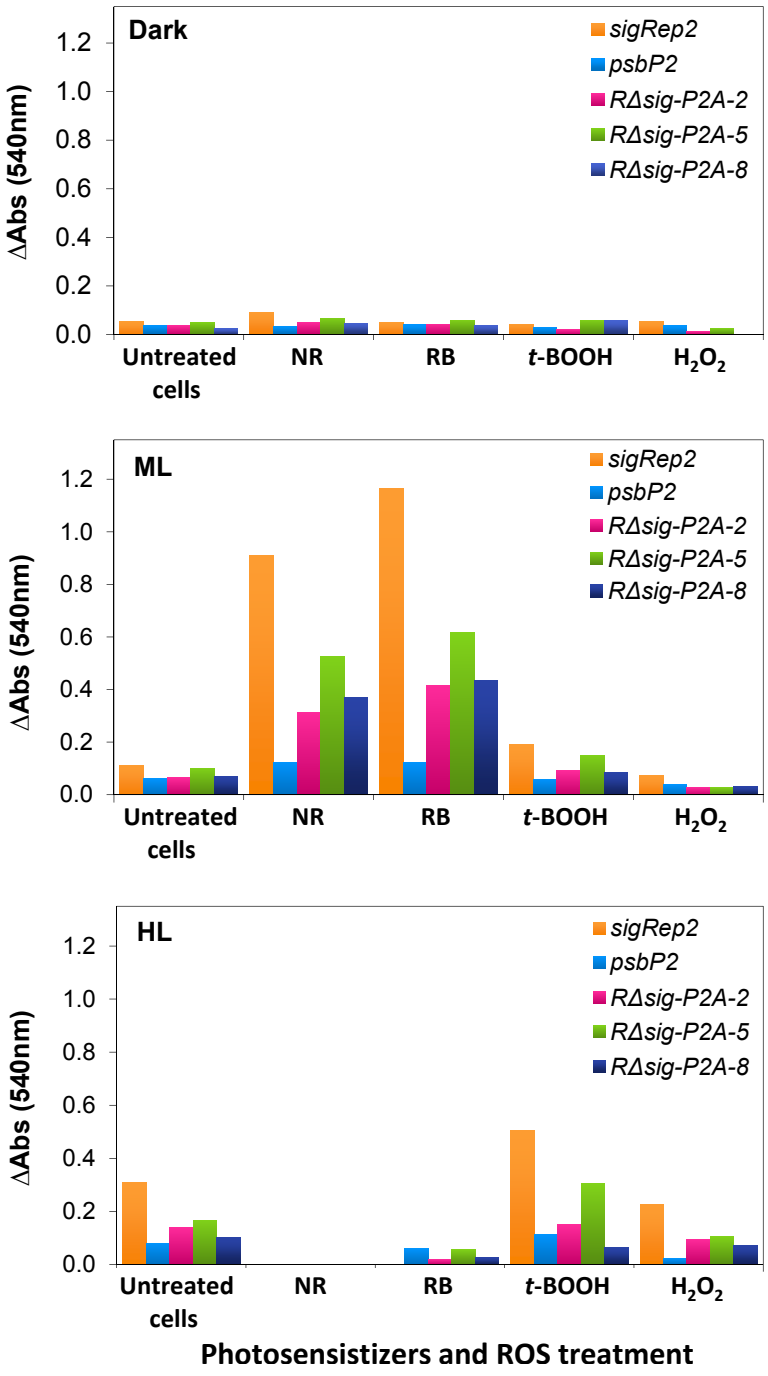

Figure 4.9. Levels of ARS2 activity determined in liquid cell cultures of *psbP2* mutant cells transformed with a copy of an intact *PSBP2* gene in T7-Arg7 plasmid. The *sigRep2*, *psbP2*, and three lines (*RΔsig-P2A-2*, *RΔsig-P2A-5*, and *RΔsig-P2A-8*) transformed with a pP2-T7Arg7 plasmid were used for the analysis. After the chemical treatment, samples were exposed to dark, ML (100 µmol photons m^{-2} s^{-1}) or HL (920 µmol photons m^{-2} s^{-1}) conditions for 8 hours. The experiment was performed once.

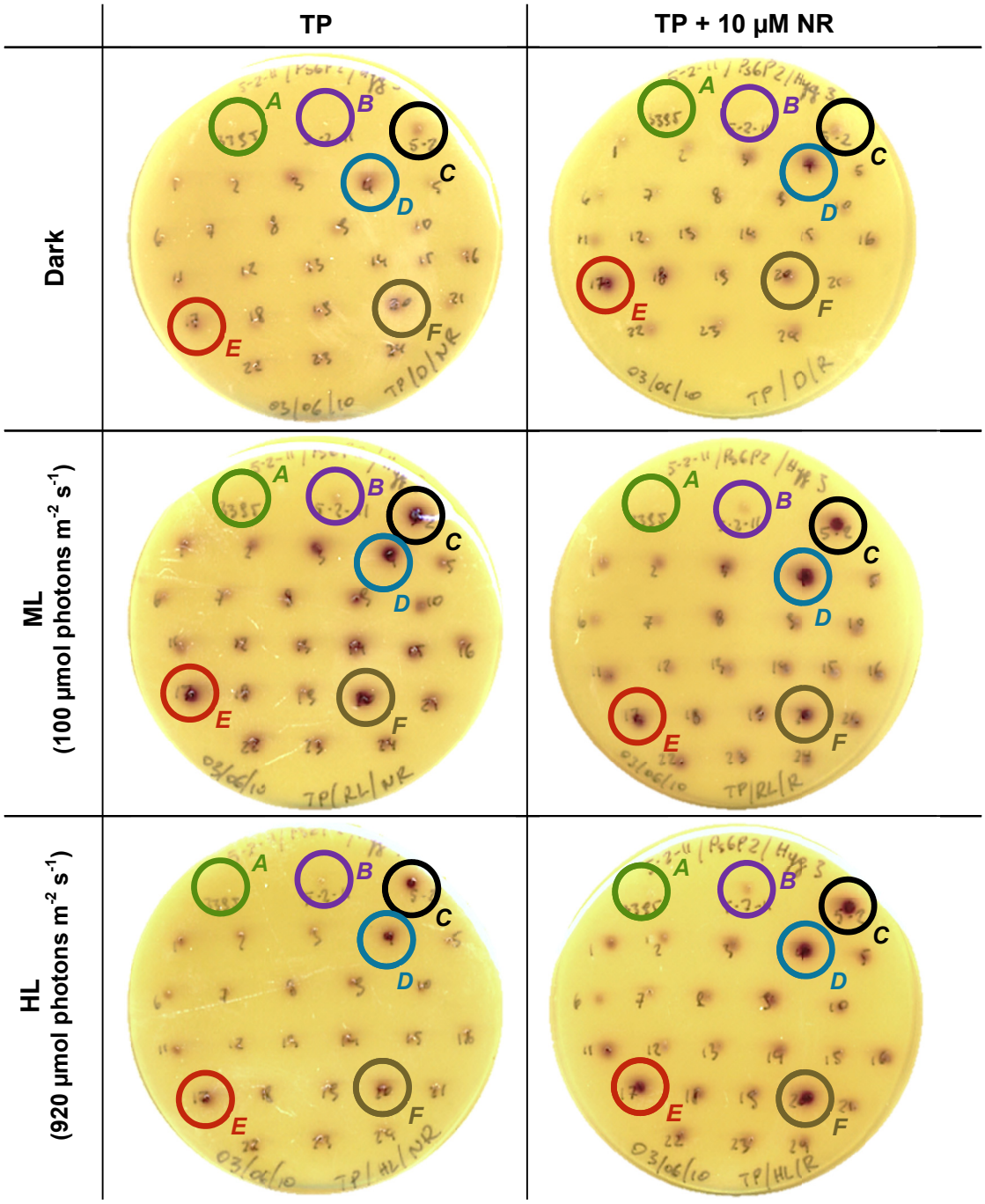

Figure 4.10. Colonies transformed with pP2-Hyg3 plasmid tested on TP plates with or without NR. Lines: *CC-3395* (***A***), *psbP2* (***B***), *sigRep2* (***C***), and lines with recovered ARS2 activity: *RΔsig-P2H-4* (***D***), *RΔsig-P2H-17* (***E***), and *RΔsig-P2H-20* (***F***) are marked in circles.

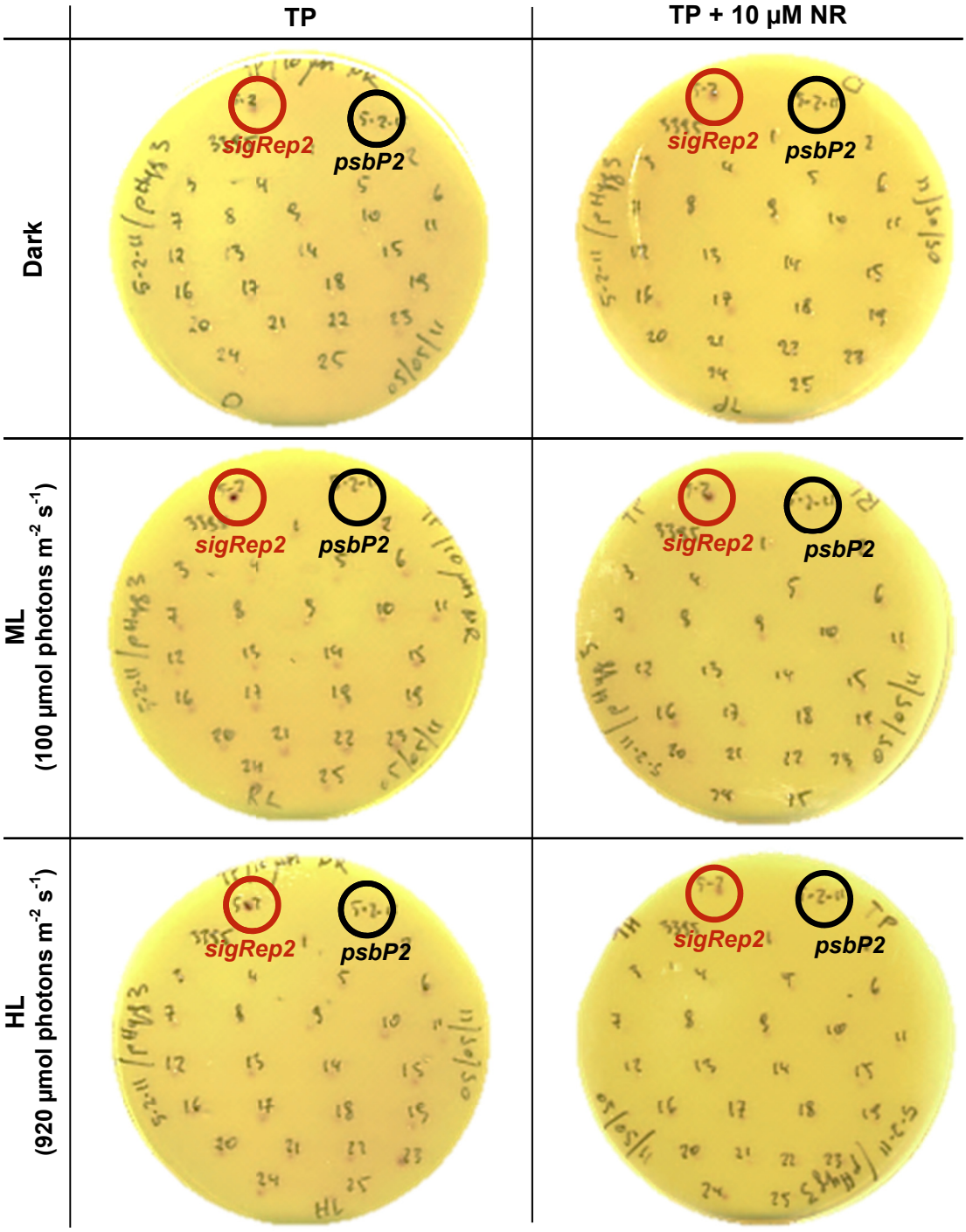

Figure 4.11. Examination of ARS2 activity in *C. reinhardtii psbP2* transformed with an empty pHyg3 plasmid. None of the 25 colonies tested showed rescued $^1O_2$-inducible signalling phenotype detectable by means of ARS2 activity; *sigRep2* was used as the positive and *psbP2* as the negative control.

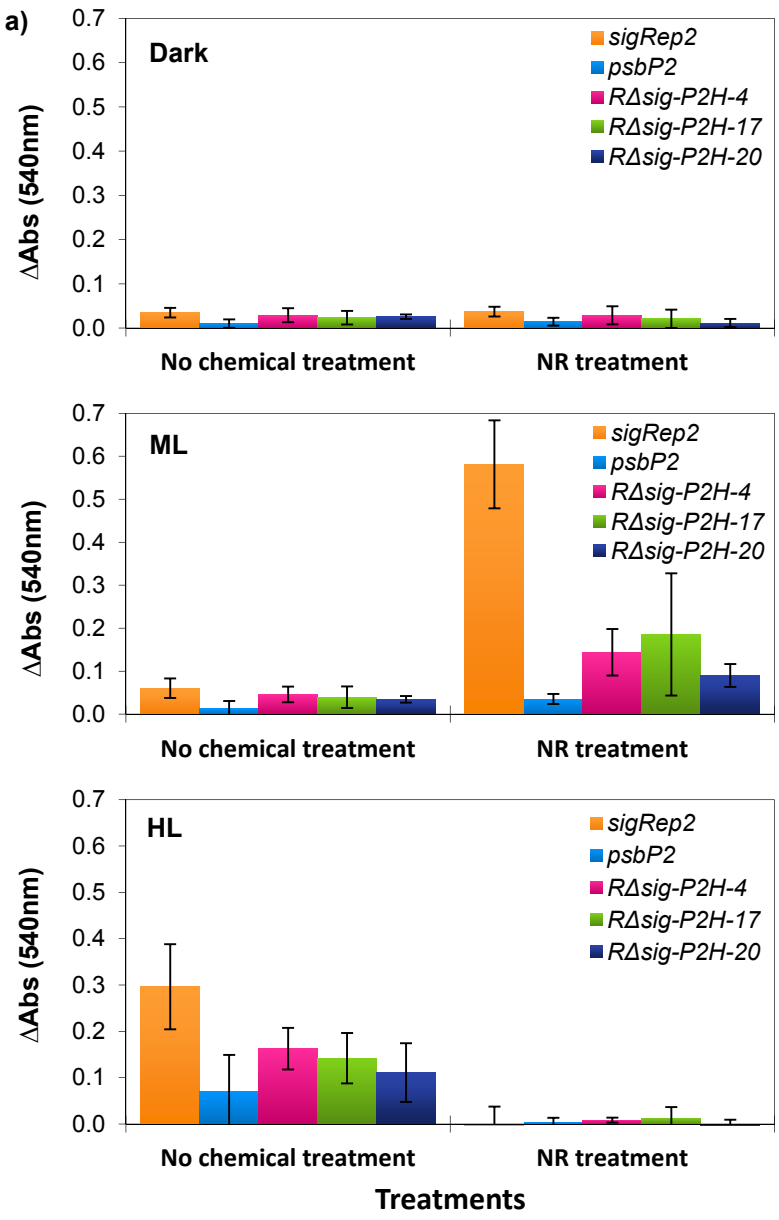

Figure 4.12. Levels of ARS2 activity determined in liquid cell cultures of *psbP2* mutant cells transformed with a copy of an intact *PSBP2* gene in pHyg3 plasmid. The *sigRep2*, *psbP2*, and three lines (*RΔsig-P2H-4*, *RΔsig-P2H-17*, and *RΔsig-P2H-20*) transformed with the pP2-Hyg3 plasmid were used for the analysis. After the chemical treatment, samples were exposed to dark, ML (100 µmol photons m^{-2} s^{-1}) or HL (920 µmol photons m^{-2} s^{-1}) for 8 hours. Data showed represents averaged results obtained from three experiments with three replicates of each sample per trial (n = 9); error bars represents the standard deviation.

conditions. Levels of *GPX5-ARS2* activity in all lines kept in the dark were very low (Figure 4.12).

Statistical analyses of results obtained for ARS2 activity (determined by absorbance measurements) revealed that in the dark treatment with NR did not have a significant effect (P = 0.465) on *GPX5-ARS2* expression. Under the same dark conditions, interaction between lines and NR treatment was also non-significant (P = 0.261), but lines were significantly different in absorbance values (P = 0.000). However, under the ML and HL, the NR treatment had a significant effect (P = 0.000) on the absorbance values, and by extension on *GPX5-ARS2* expression. Similarly, lines from ML and HL conditions differed significantly in measured absorbance values (P = 0.000) and interaction between lines and NR treatment was also significant (P = 0.000) under these light conditions. A detailed report from ANOVA analyses is presented in Table D9.1.

4.3.6. The psbP2 mutation does not affect cell growth

The growth responses of *psbP2* and *psbP1* (*oee2*, *CC-4142*) mutants to different light intensities when grown on TAP or TP are shown in Figure 4.13. *PsbP1* failed to grow under HL conditions on either TAP or TP which is indicative of its high-light sensitivity. The growth rate was lower in this line when kept on TP media under ML conditions when compared to growth on TAP media supplemented with acetate as a reduced carbon source. *PsbP2*, unlike *psbP1*, appears to grow at a similar rate to the *sigRep2* or parental *CC-3395* strain on either TAP or TP media and under the different light conditions examined (Figure 4.13). Thus, the *psbP2* mutant does not exhibit a light-dependent growth phenotype nor is it sensitive to high-light exposure.

4.3.7. Photosynthetic characteristics of psbP2

Results from the experiment, to compare sensitivities of *psbP2* and *psbP1* (*oee2*, *CC-4142*) mutant lines to HL or their overall ability to photosynthesize, showed that these two lines displayed different phenotypic characteristics (*Section 4.3.6*, Figure 4.13). To further examine whether the *psbP2* mutation altered photosynthesis efficiency and by

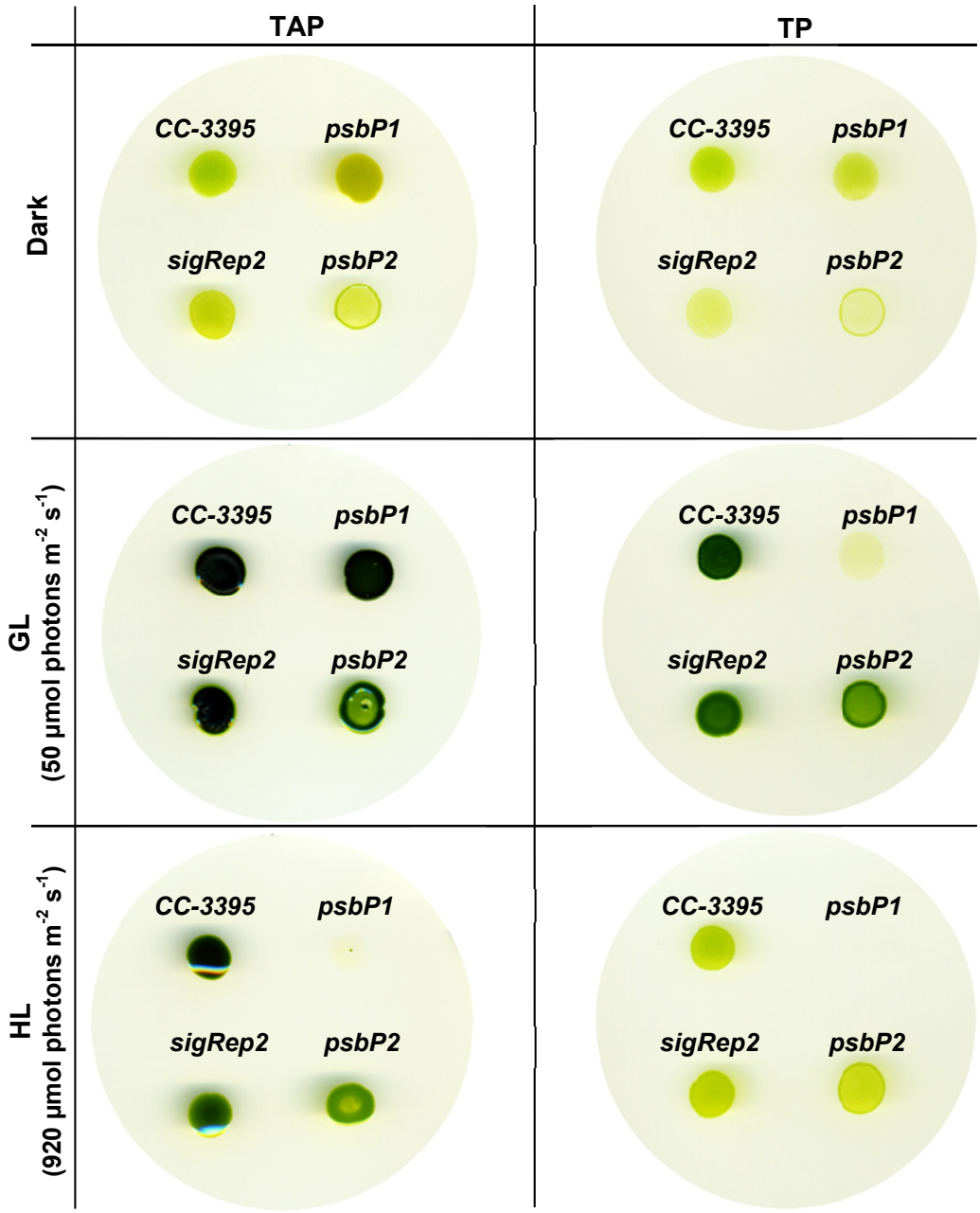

Figure 4.13. An examination of the growth response of the *psbP2* mutant to different light intensities. *CC-3395*, *sigRep2*, *psbP2* and *CC-4142* (*oee2*, denoted as *psbP1* on the Figure) were grown in TAP medium under GL conditions until mid-log phase 3-5 x 10^6 cells mL^{-1}, harvested by centrifugation, and resuspended in TP, normalizing cell concentration of each line to 5 x 10^7 cells mL^{-1}. Aliquots of 10 µL were spotted onto TAP or TP plates and exposed to dark, GL or HL conditions. Colonies were examined and photographed after 5 days.

these means thus affects $^1O_2$-inducible signalling indirectly (e.g. by reduced $^1O_2$ generation) the photosynthetic characteristics of the *psbP2* line were examined using pulse-amplitude modulated chlorophyll fluorescence.

Photosynthetically, *psbP2* and *sigRep2* responded similarly to changes in light intensity (Figure 4.14). *SigRep2* appeared to exhibit marginally higher levels of Q_A reduction at light intensities above 250 μmol photons m^{-2} s^{-1} when compared to *psbP2* (Figure 4.14a). Similarly, when capacity of the two strains to perform non-photochemical quenching was compared, there was a marginal trend towards higher NPQ in the *sigRep2* strain (Figure 4.14b).

To determine if the PSBP2 protein was involved in PSII stability or repair, as had been shown for the PPL1 protein of *A. thaliana* (Ishihara *et al.* 2007), the response to photoinhibitory light conditions, followed by a short recovery period, was examined in *psbP2* and *sigRep2*. Under normal growth conditions, *psbP2* had a consistently lower F_v/F_m measurement of 0.583 ± 0.011 compared to 0.630 ± 0.030 for *sigRep2*. During exposure to the photoinhibitory treatment at 1200 μmol photons m^{-2} s^{-1} the measured F_v/F_m of *psbP2* decreased faster than that of *sigRep2* (Figure 4.15). This difference was also observed when the two cultures were treated with chloramphenicol to disrupt PSII repair (Figure 4.15). However, both strains rapidly recovered when placed in low-light (20 μmol photons m^{-2} s^{-1}), with F_v/F_m levels reaching or exceeding the starting values within 30 min. For both strains, the recovery appears to be completely due to the PSII synthesis-repair cycle, because no recovery occurred in the chloramphenicol treated cultures (Figure 4.15).

4.3.8. The PSBP1 and PSBP2 proteins are not interchangeable in C. reinhardtii

Because PSBP1 and PSBP2 belong to the same family of proteins that share a sequence similarity of the PSBP domain, it was examined whether the PSBP1 protein can complement the abrogated $^1O_2$-inducible retrograde signalling in the *psbP2* mutant line. Thus, the wild-type *PSBP1* gene was isolated from the *CC-3395* line and introduced into the pHyg3 vector (Berthold *et al.* 2002). This produced pP1-Hyg3 which was used to transform the *psbP2* line. Similarly, the *psbP1* strain was transformed with

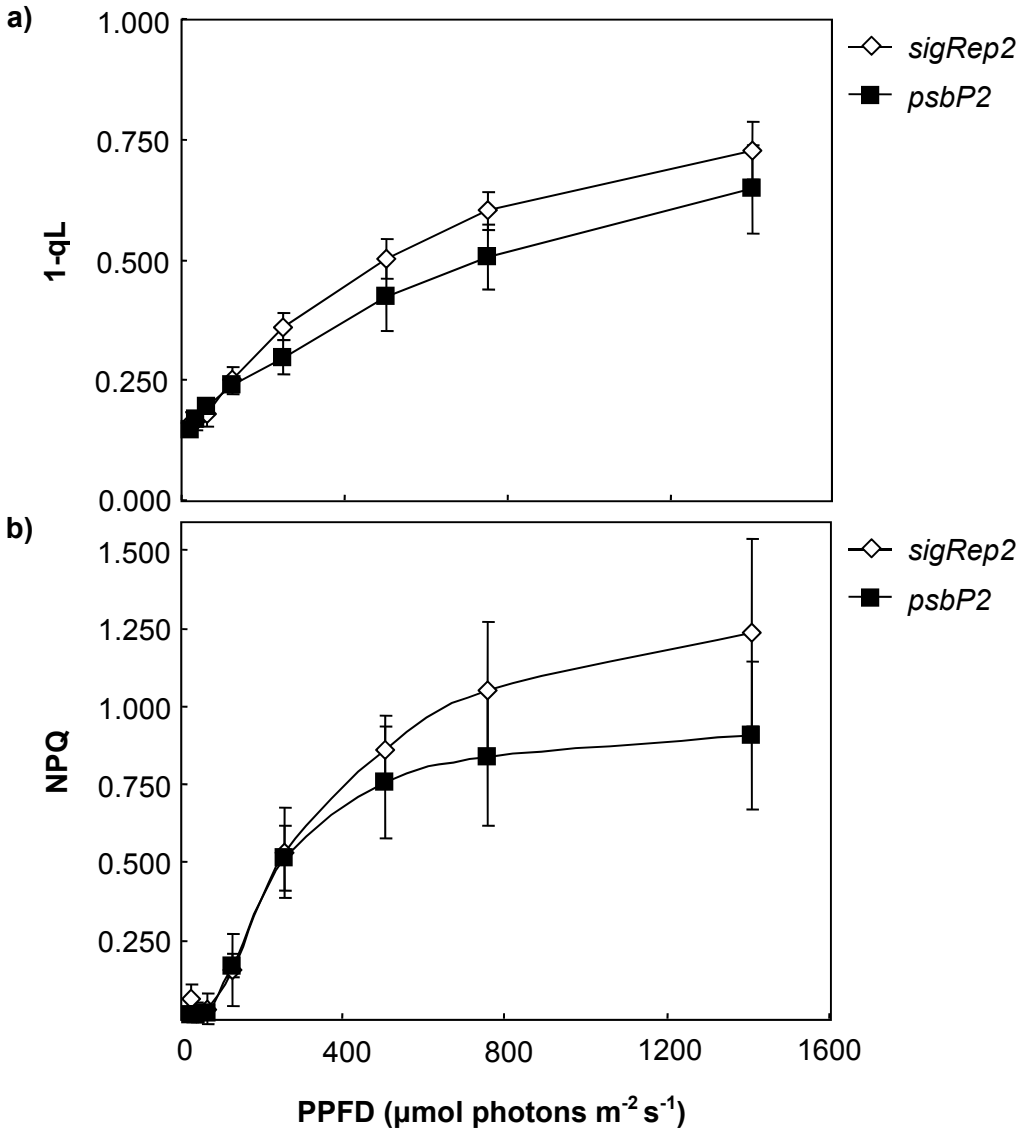

Figure 4.14. Steady-state photosynthesis in *sigRep2* and *psbP2*. As described in the Materials and Methods section, cultures were dark adapted and then exposed to increasing levels of light. The relative reduction state of Q_A is represented by 1-qL (**a**), while the ability of the cell to dissipate excess light energy as heat is represented by NPQ (**b**). Values represent means ± SE (n = 3); data and the Figures were provided by Dr. Ken Wilson.

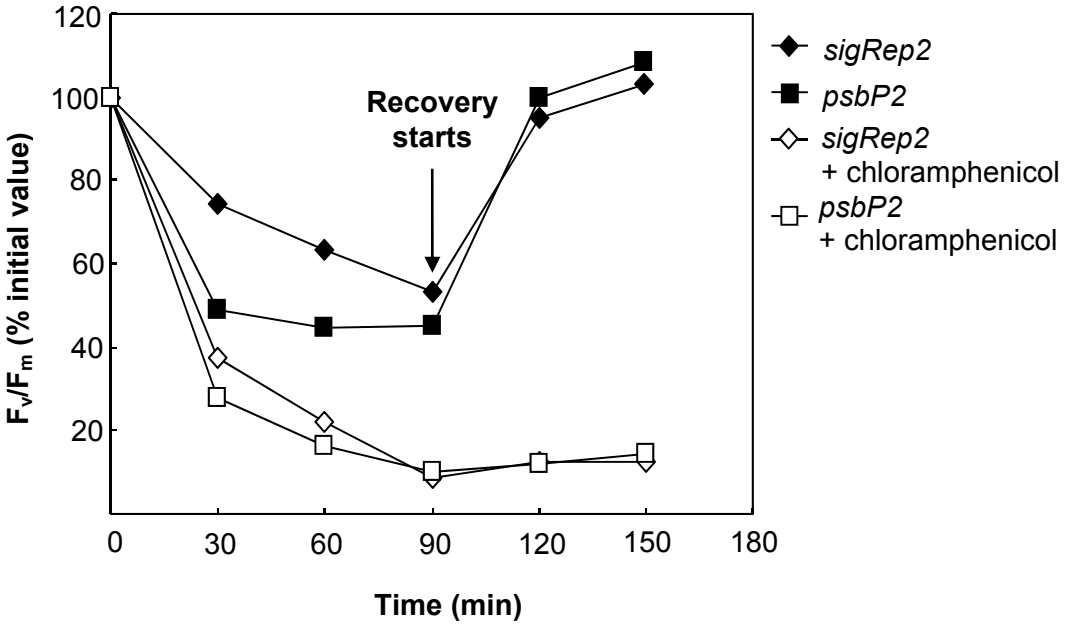

Figure 4.15. Sensitivity of *psbP2* to photoinhibition. Cultures were exposed to 1200 µmol photons m^{-2} s^{-1} in the presence or absence of chloramphenicol, for 90 min followed by a recovery at 20 µmol photons m^{-2} s^{-1}. The experiment was performed once; data and the Figure were provided by Dr. Ken Wilson.

pP2-Hyg3. Following selection on hygromycin B, *psbP2* transformants were tested for *GPX5-ARS2* inducibility in response to exogenous $^1O_2$, while the *psbP1* strain was tested for the ability to grow on TP media in the light.

As shown in Figure 4.16, slightly elevated ARS2 activity in *psbP2* transformed with pP1-Hyg3 is at a lower levels than in *sigRep2*, which was used as a positive control and much lower than levels observed in lines rescued with pP2-T7Arg7 (Figures 4.8) or pP2-Hyg3 in particular (Figure 4.10). ARS2 activity levels in *RΔsig-P2H-4*, *RΔsig-P2H-17*, and *RΔsig-P2H-20* were as strong or even exceeded levels observed in *sigRep2* on plates (Figure 4.10). Additionally, ARS2 activity observed in *psbP2* transformed with pP1-Hyg3 is at the similar levels to these that were observed in control transformation with an empty pHyg3 vector (Figure 4.11). Therefore, it can be concluded that the pP1-Hyg3 vector was not able to restore $^1O_2$-dependent ARS2 activity in the *psbP2* strain (Figure 4.16), despite the fact it could rescue HL sensitivity of the *psbP1* strain (Figure 4.17), although additional experiments could be required. In a similar fashion, the pP2-Hyg3 vector was unable to restore the HL lethal phenotype of the *psbP1* strain (Figure 4.18).

4.3.9. Acclimation to ROS stress in the psbP2 mutant line

Acclimation to stress caused by high-light, lower or higher temperatures, wounding or relatively low levels of ROS increases the tolerance of plants to a subsequent more severe stress condition (Chang *et al.* 2004; Karpinski *et al.* 1999; Ledford *et al.* 2007; Orozco-Cardenas *et al.* 2001). Figure 4.19 shows the results obtained from an acclimation-response analysis of *psbP2* to $^1O_2$ exposure. None of the non-pretreated lines were able to survive a challenge with the RB concentrations tested, regardless of whether they were grown on TAP or TP. On the contrary, all lines pre-treated with a sub-lethal, 2 μM RB stress, clearly showed an acclimation response to $^1O_2$ and a higher rate of survival on TAP than on TP. Although *CC-3395* displayed a slightly lower tolerance to RB exposure than *sigRep2*, *RΔsig-P2H-17*, *RΔsig-P2H-20*, or even *psbP2* on TAP (lethal toxicity of 16 μM RB), as well as all the other lines examined on TP (lethality already at 8 μM RB), it was assumed that this difference is of no biological

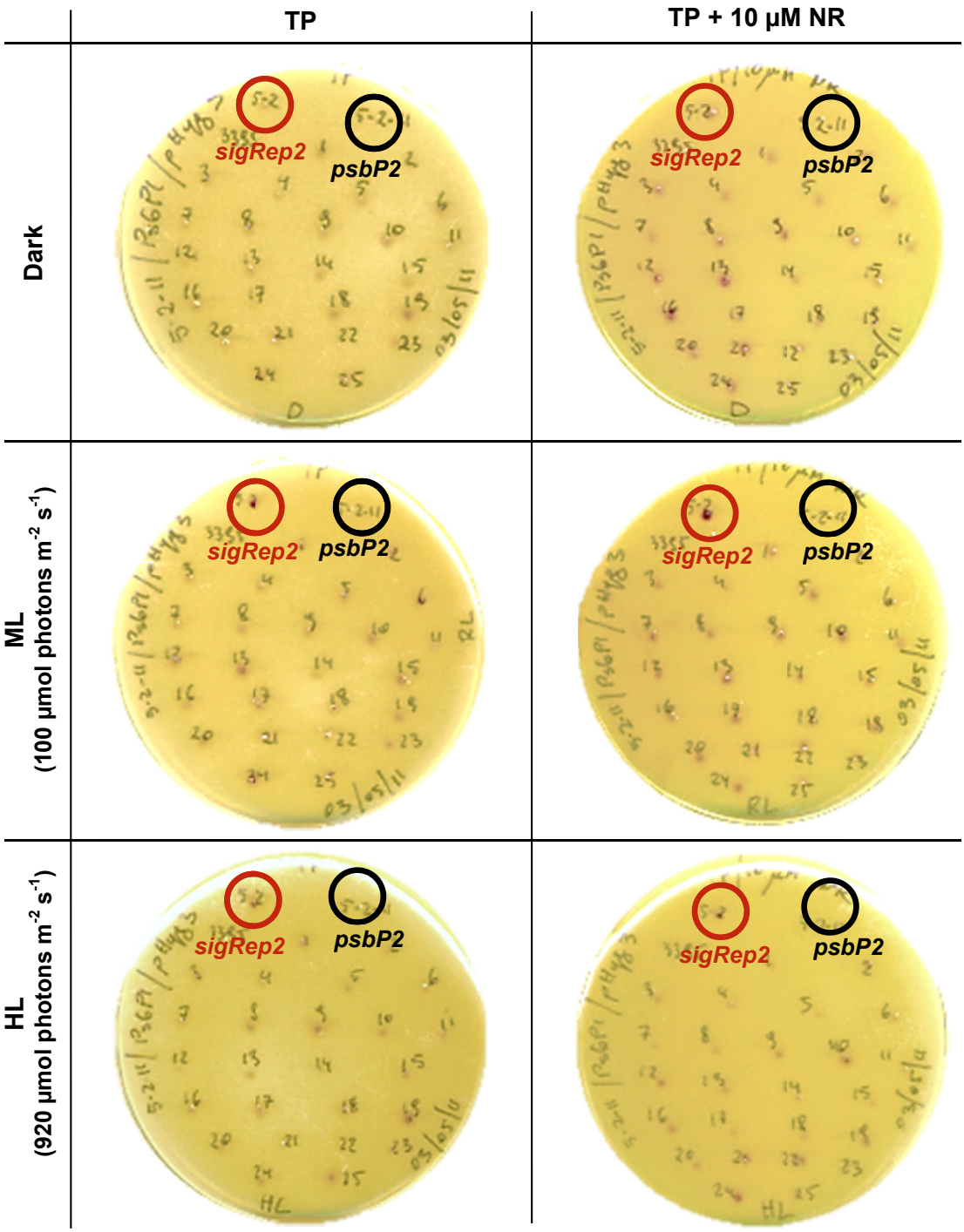

Figure 4.16. Examination of ARS2 activity in *C. reinhardtii psbP2* transformed with pP1-Hyg3 plasmid. None of the 25 colonies tested showed rescued $^1O_2$-inducible signalling phenotype detectable by means of ARS2 activity; *sigRep2* was used as the positive and *psbP2* as the negative control.

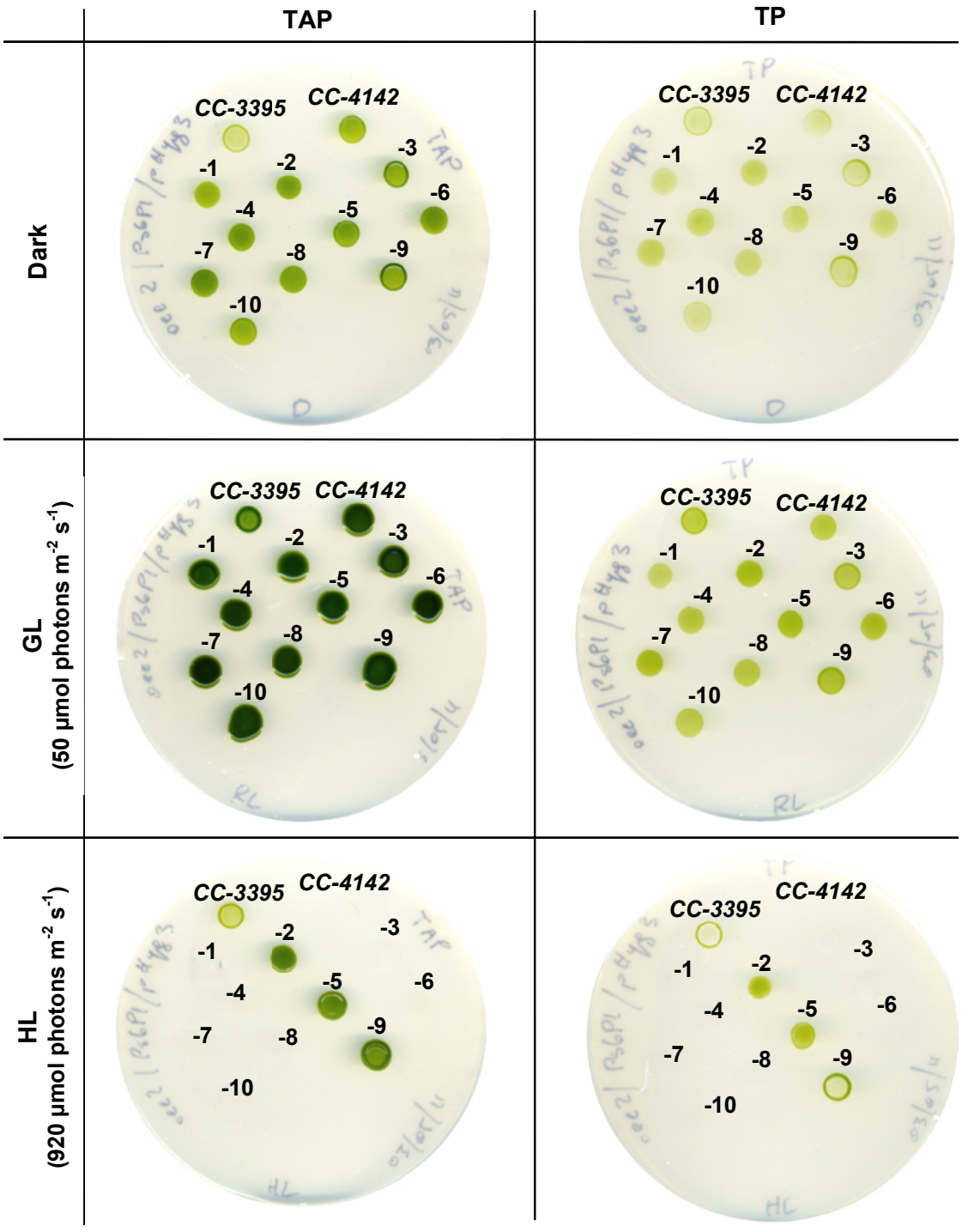

Figure 4.17. *C. reinhardtii CC-4142* (*psbP1*) mutant line transformed with the pP1-Hyg3 plasmid. Three out of ten (~33%) of examined lines displayed rescued wild-type phenotype manifested as the ability to survive HL conditions when grown either on TAP or on TP; *CC-3395* was used as the positive and *CC-4142* as the negative control.

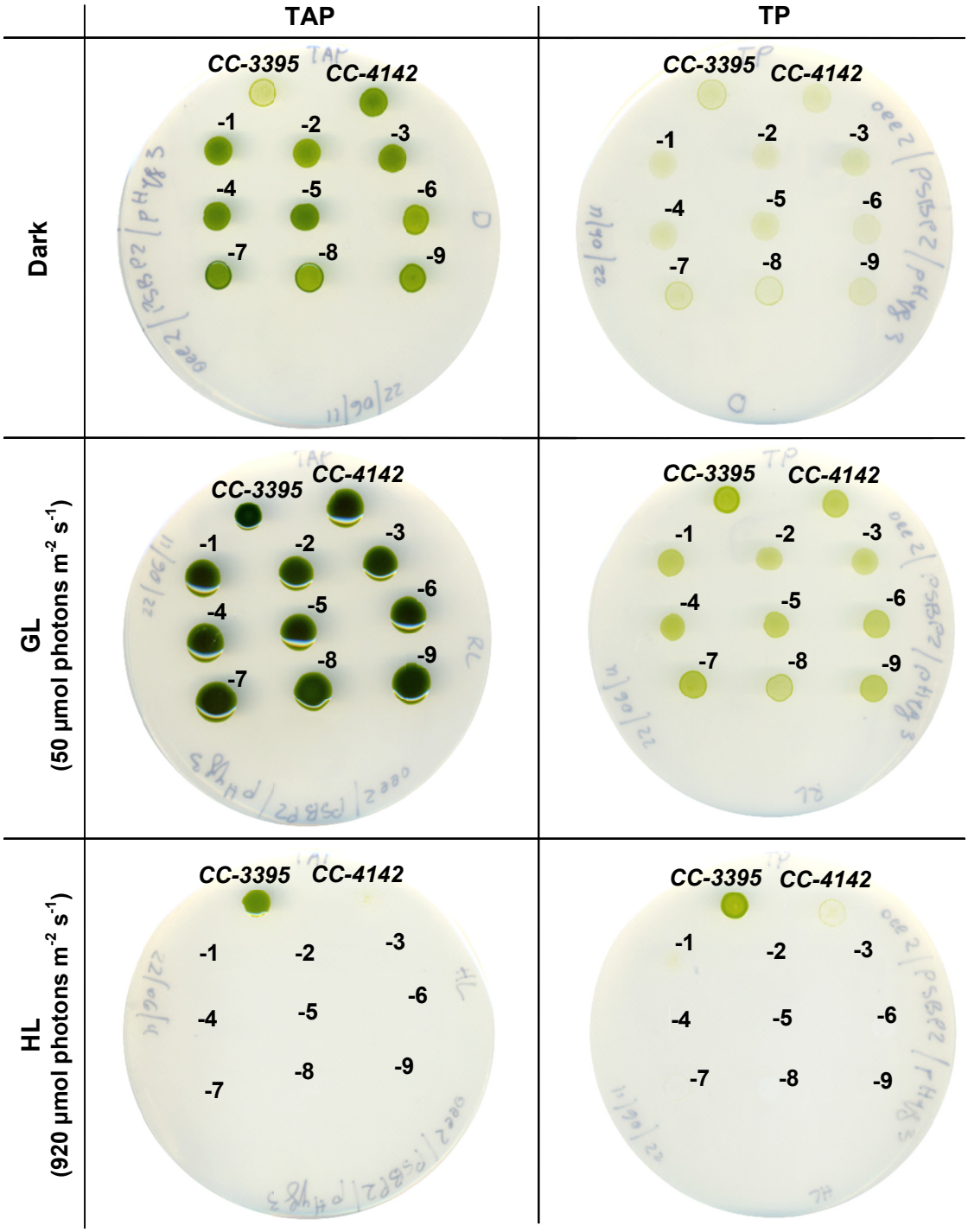

Figure 4.18. *C. reinhardtii CC-4142* (*psbP1*) mutant line transformed with the pP2-Hyg3 plasmid. None of the transformed cells showed rescued photosynthetic phenotype; *CC-3395* was used as the positive and *CC-4142* as the negative control.

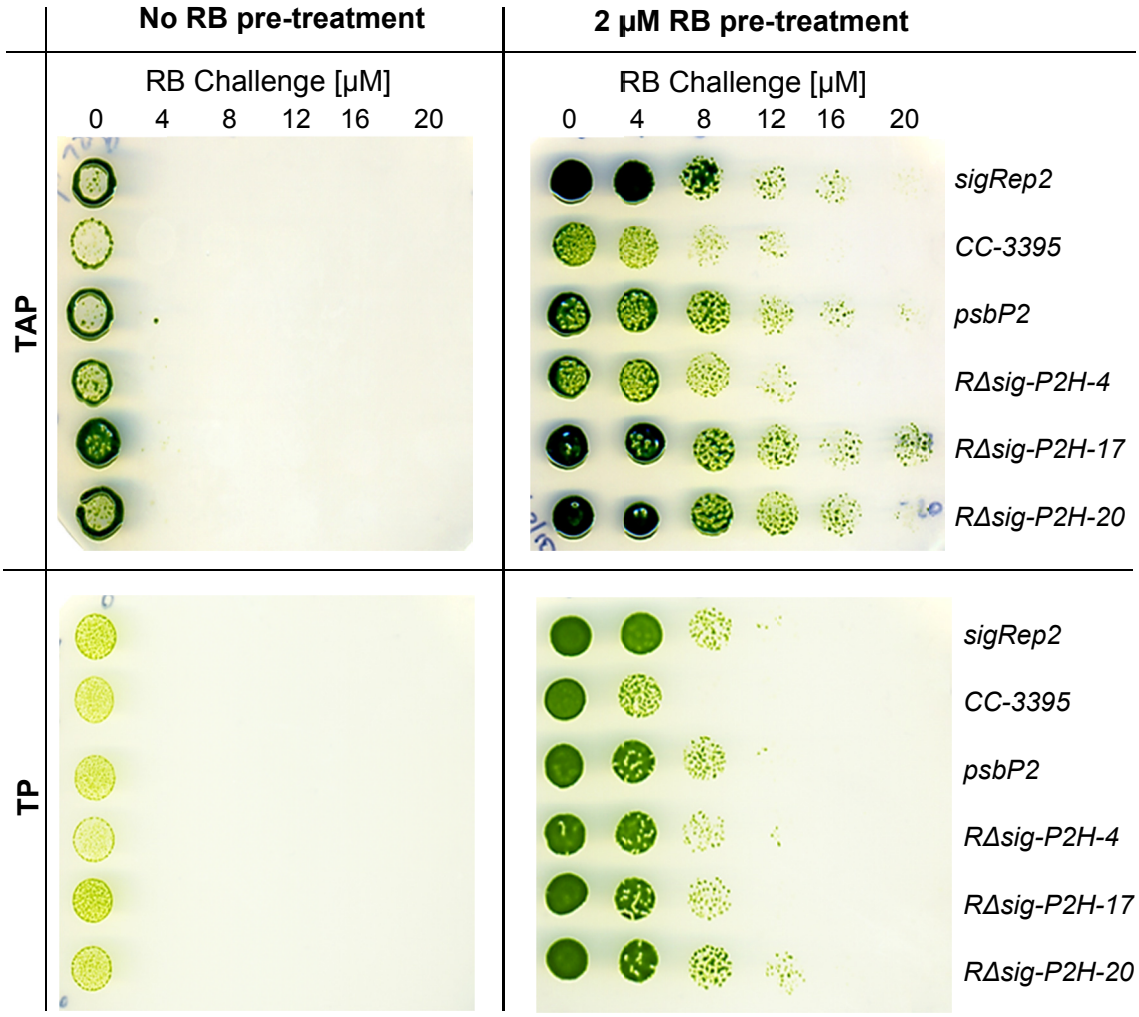

Figure 4.19 Acclimation to singlet oxygen stress in *sigRep2*, *CC-3395*, *psbP2*, and three lines (*RΔsig-P2H-4*, *RΔsig-P2H-17*, and *RΔsig-P2H-20*) rescued with the genomic version of the *PSBP2* gene in the pHyg3 plasmid. Any concentration of RB challenging cells with $^1O_2$ was shown to be lethal in non-acclimated lines grown either on TAP or TP. Lines pre-treated with 2 µM RB displayed acclimation to different concentrations of RB and thus levels of $^1O_2$ produced. Acclimated cells showed better growth/survival rate on TAP than TP.

significance or could be caused by factors not directly related to the subject of the experiment.

4.3.10. Analysis of GPX5 and GPX5-ARS2 reporter gene expression in psbP2 by sqRT-PCR

Two independent transcription start sites in the *GPX5* gene sequence were shown by Fischer *et al.* (2009) to be differentially active (Figure 4.20). To determine if the native *GPX5* gene was being regulated in the same manner as the *GPX5-ARS2* reporter gene, the relative transcript abundance of the long and short forms of each gene were examined using semi-quantitative RT-PCR (sqRT-PCR). The transcript levels were compared between cultures grown under GL or treated with 0.5 μM RB and returned to GL.

In an assessment of the transcript levels examined by sqRT-PCR it is necessary to emphasize that primers specific for a long version of the transcript amplify only native *GPX5* promoter region. However, primers designed to align with the short version of the transcript amplify both long and short versions of the native *GPX5*.

As one can see in Figure 4.21, the sqRT-PCR produced a 103 bp DNA fragment (amplification with primers KW235 and KW236) of the short version of the native *GPX5* transcripts (Fischer *et al.* 2009; Figure 4.21a), which accumulated at lower levels compared to *RΔsig-P2H-4* from GL than in *CC-3395* and *sigRep2*, but lower in *RΔsig-P2H-4* than in *psbP2* from the same treatment conditions (Figure 4.21a). The *psbP2* line accumulated lower levels of short *GPX5* transcript than *sigRep2* but similar to *CC-3395* (Figure 4.21a). Differences in short *GPX5* transcripts were more pronounced in lines treated with RB and returned to GL conditions (Figure 4.21a), with *psbP2* and *RΔsig-P2H-4* accumulating short *GPX5* at lower levels than *CC-3395 or sigRep2* (Figure 4.21a).

When the transcript levels of the long version of the endogenous *GPX5* (Fischer *et al.* 2009; amplification with KW233 and KW234 primer producing 102 bp fragment; Figure 4.21b) were compared, expression was at similar levels in *CC-3395*, *sigRep2*, and *psbP2*, both from GL and RB treatment, but lower in *RΔsig-P2H-4* in case of both treatment conditions (Figure 4.21b).

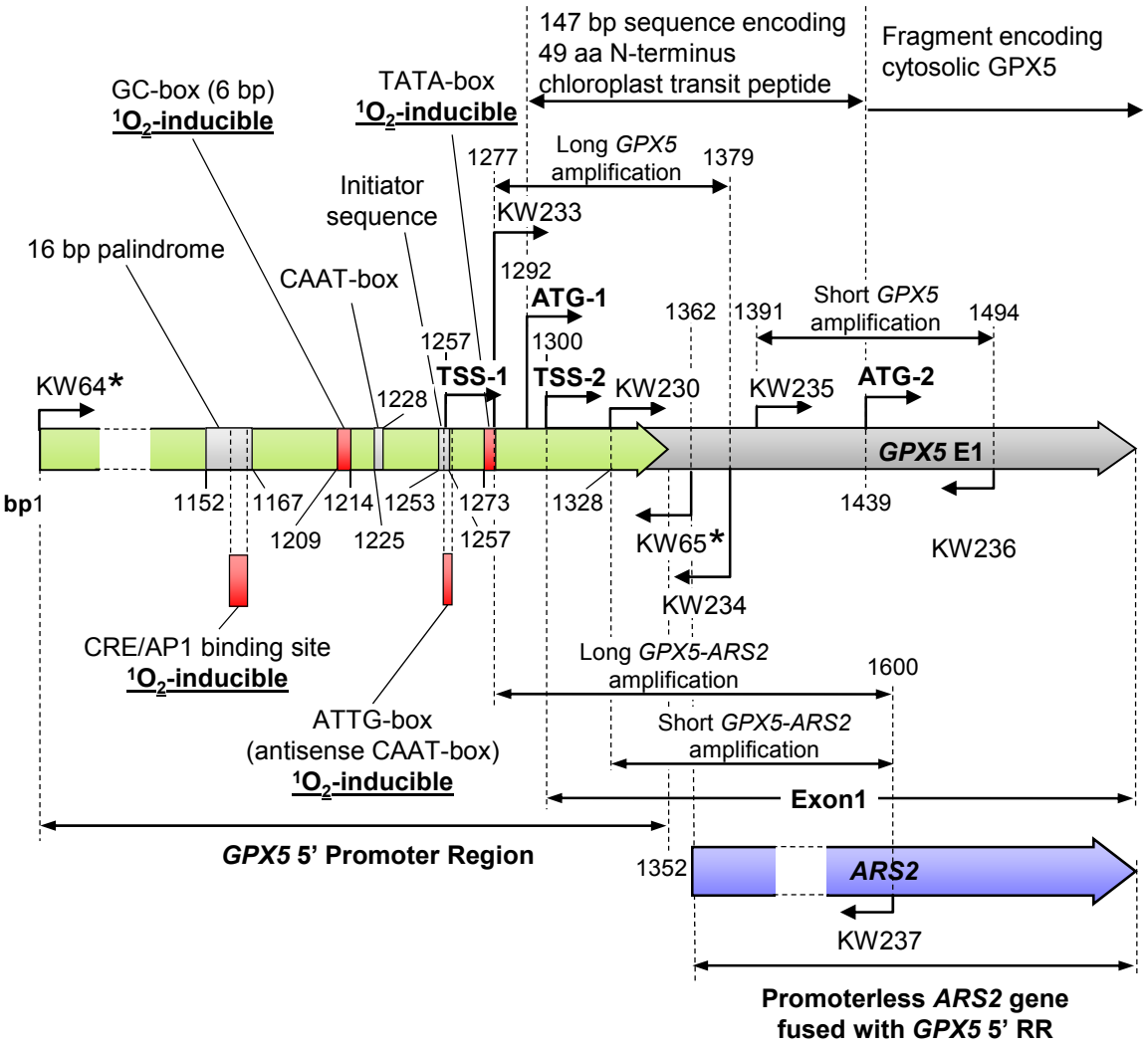

Figure 4.20. A model of the *GPX5* 5' regulatory region indicating short and long version of the transcript. Two independent transcription start (TSS-1 and TSS-2) and two independent translation start sites are indicated (ATG-1 and ATG-2). The TSS-1 is located in the core initiator sequence and TSS-2 5 bp downstream from the ATG-1. The two transcription start sites are responsible for initiation of transcription of the same *GPX5* gene but with two transcript versions, encoding protein with the chloroplast signalling sequence or truncated version encoding cytosol-localized GPX5. The model was constructed based on genomic DNA sequence obtained from JGI (DOE Joint Genome Institute, www.phytozome.net) and *cis*-regulatory elements characterized by Fischer *et al.* (2009) as being required for transcriptional activation of *GPX5* by $^1O_2$. Sequences were mapped and model was constructed using Vector NTI 11 software.

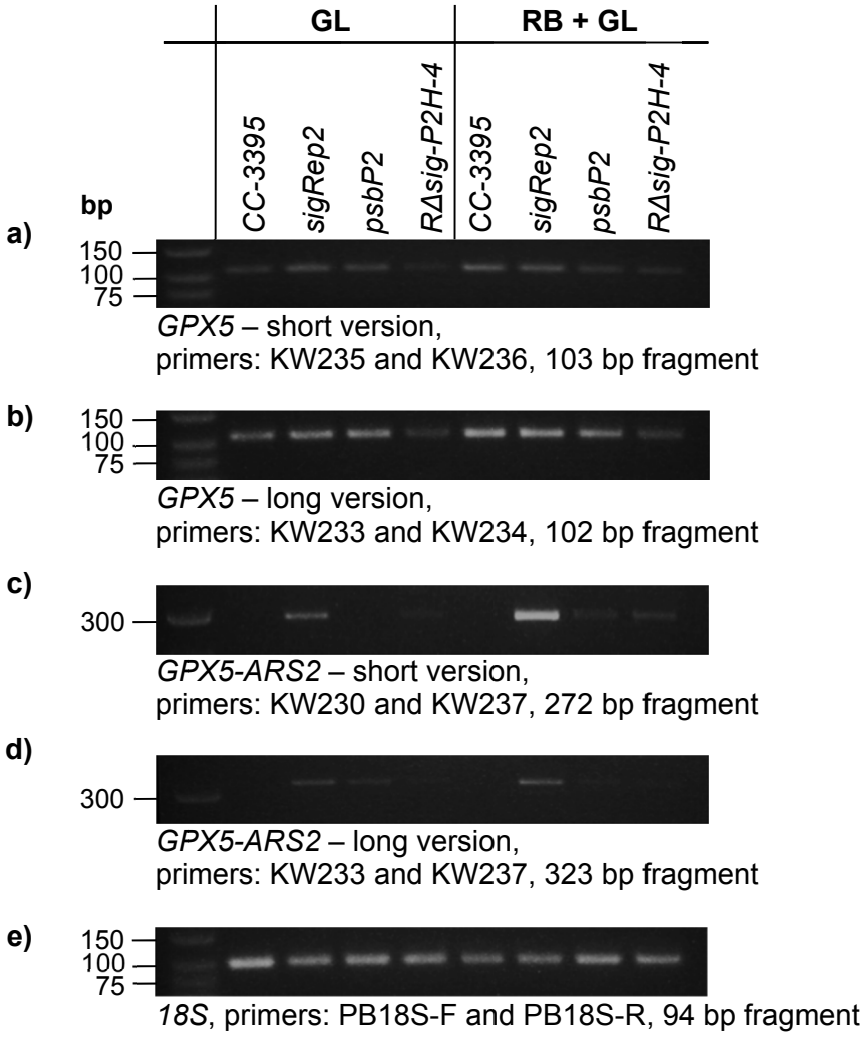

Figure 4.21. The sqRT-PCR-based analysis of the *GPX5* and *GPX5-ARS2* transcript levels. **a)** Short (primers KW235 and KW236) and **b)** long (primers KW233 and KW234) versions of the *GPX5* gene; **c)** short (primers KW230 and KW237) and **d)** long (primers KW233 and KW237) versions of the *GPX5-ARS2* reporter gene in *CC-3395*, *sigRep2*, and *psbP2,* and *RΔsig-P2H-4*. **e)** sqRT-PCR amplification of the *18S* rRNA was used as a loading control. Annealing sites for primers used for short and long *GPX5*, and short and long *GPX5-ARS2* are depicted in Figure 4.20.

Transcripts abundance of the introduced *GPX5-ARS2* reporter gene corresponding to the short version of the *GPX5* promoter region fused with *ARS2* (272 bp; Figure 4.21c), accumulated at a higher level in *sigRep2* than in *RΔsig-P2H-4* from the GL, while the short *GPX5-ARS2* fusion transcript could not be detected in *psbP2* under this treatment condition (Figure 4.21c). The *sigRep2* line expressed the short *GPX5-ARS2* fusion transcript at higher levels than *psbP2* and *RΔsig-P2H-4* when exposed to RB treatment (Figure 4.21c). As expected, no transcript levels for the short *GPX5-ARS2* fusion were detected in *CC-3395* line (Figure 4.21c).

In case of the long *GPX5-ARS2* fusion transcript (323 bp; Figure 4.21d), the expression from GL was higher in *sigRep2* than in *psbP2* but in the same time higher in *psbP2* than in *RΔsig-P2H-4* (Figure 4.21d). The RB exposure induced higher expression of the long *GPX5-ARS2* fusion in *sigRep2*, while the expression levels in *psbP2* and *RΔsig-P2H-4* were very low (Figure 4.21d). Again, as expected, no transcripts for the long *GPX5-ARS2* were observed in the *CC-3395* line (Figure 4.21d).

4.4. Discussion

Using the RESDA-PCR protocol of Gonzalez-Ballester *et al.* (2005), an attempt was undertaken to determine the plasmid insertion sites of three secondary mutants which were unable to demonstrate ARS2 activity following HL exposure or treatment with a photosensitizer. From the three lines tested, the insert site was determined only for *ΔsigRep2-11*. The mutagenized gene responsible for the *ΔsigRep2-11* phenotype was identified as *psbP2*, followed by examination how the protein encoded by this gene could play a role in $^1O_2$-dependent control of *GPX5-ARS2* activity, and by extension *GPX5* expression. Although the results of the RESDA-PCR protocol identified the insertion site in the 5' UTR of the predicted *PSBP2* gene, sqRT-PCR analysis showed that the gene was still being transcribed (Figure 4.4), although at lower levels when compared to the founder *CC-3395* line. The transcript in *psbP2* was shown by 5' RACE to be defective (Figure 4.5). It was possible to confirm that the *PSBP2* gene played a role in regulating the *GPX5* driven ARS2 activity by the partial rescue of the $^1O_2$-dendent signalling phenotype with a genomic copy of the *PSBP2* gene. A neighbouring gene (sensor histidine kinase-related, locus Cre16.g678900), labelled as a putative 2-component

regulatory receptor, is more likely to be involved in signal transduction, based on its bioinformatic analysis, however, it was demonstrated that the gene is intact and is transcribed normally.

With the identification of the *psbP2* mutant, that appears to disrupt the expression of the *GPX5-ARS2* reporter gene, it was demonstrated that the *sigRep* strains can be used to probe the molecular genetics of the $^1O_2$-dependent chloroplast-to-nucleus retrograde signalling pathway. Although the sqRT-PCR analysis indicated that the insertional mutation did not completely abrogate expression of the *psbP2* (Figure 4.4a), this could be indicative that a fully functional protein cannot be synthesized. Furthermore, as it was determined by 5'RACE, the insertional event disrupted the 5' UTR of the *PSBP2* gene resulting in the production of truncated transcripts, further supporting the hypothesis that it cannot encode a functional protein (Figure 4.5). Because RESDA-PCR was designed to amplify only the sequence adjacent to 3'-end of the Ble^R in pSP124 plasmid, only this fragment of the insert was sequenced and analyzed. However, this should be further examined by sequencing of the both ends of the pSP124s insert, which may provide additional information regarding the size and the exact location of the insertion and thus, clarify its effect on *PSBP2* expression.

Bioinformatic analyses suggested that the PSBP2 protein has a Tat signal peptide which should target the protein to the thylakoid lumen in the chloroplast. Thus, it would appear that the PSBP2 protein could be part of a very early step in the $^1O_2$-dependent chloroplast to nucleus retrograde signalling pathway. It is possible that the insertion in 5'UTR may have disrupted the targeting sequence and thus abolished transport of the psbP2 to the chloroplast.

To verify involvement of the PSBP2 protein in a $^1O_2$-inducible retrograde signalling pathway, an attempt was undertaken to rescue the signalling phenotype in the *psbP2* mutant line. A PCR-amplified 3626 bp fragment of genomic DNA including the *PSBP2* coding sequence, 1423 bp upstream of its predicted transcription start site, and 437 bases of predicted 3'UTR was reintroduced into the *psbP2* strain to rescue the signalling phenotype. Following transformation with either the pP2-T7Arg7 vector or the pP2-Hyg3 vector, approximately 40% of the transformed colonies exhibited $^1O_2$-inducible ARS2 activity. However, based on the semi-quantitative analysis, ARS2

activity in the rescued lines was never greater than around 40% of that observed in the *sigRep2* line, as determined by absorbance measurements. On the other hand, based on the repeatability of the system, using different vectors and the lack of any level of rescue in cells transformed with the empty vector, the results strongly suggest that the mutation in the *PSBP2* gene was responsible for abrogated $^1O_2$-signalling.

While the lack of 100% restoration of the signalling phenotype following gene rescue is disconcerting, there are a number of possible reasons which could explain the phenomenon. One possibility is due to positional effects following the insertion of the vector construct. *C. reinhardtii* is known to effectively silence transgenes following transformation (Cerutti *et al.* 1997a, b; Jeong *et al.* 2002; Wu-Scharf *et al.* 2000), thus methylation and chromosome packaging may be factors limiting the expression. A second possibility is that the 3626 bp fragment used to attempt to rescue the signalling phenotype is incomplete. A regulatory motif at either the 5' or 3' end of the gene may be missing. This is perhaps the most likely reason for the repeatable partial rescue. To address this issue in the future, a longer fragment will need to be obtained from a genomic library, as PCR amplification of long genomic sequences from *C. reinhardtii* is notoriously difficult. Alternatively, native *PSBP2* regulatory region could be substituted with one with high constitutive activity such as *PSAD* (Fischer and Rochaix 2001) used in this study, truncated *RBCS2* (Fuhrmann *et al.* 2004; Lumbreras *et al.* 1998) or *HSP70A*, separately or in tandem with addition of intron 1 of *RBCS2* to enhance the expression (Sizova *et al.* 2001).

With emerging evidence indicating that the mutation in the *PSBP2* gene is responsible for the loss of a signalling phenotype observed in the *ΔsigRep2-11* strain, it leaves a question as to whether the phenotype is due to a loss of signalling or the decreased production of $^1O_2$ in cells, due to a disruption in photosynthesis. To test the possible involvement of PSBP2 in photosynthetic processes, the overall photosynthetic performance and HL sensitivity on TAP and TP plates were examined. The *psbP2* mutant is capable of photoautotrophic growth under HL, suggesting that the overall photosynthetic capacity of the mutant is relatively unaffected. To probe this at a more physiological level, pulse-amplitude modulated chlorophyll fluorescence was used to examine the functionality of PSII and the electron transport chain. The relatively high

level of F_v/F_m and the lack of a difference in PSII redox state under steady-state light conditions, as estimated by 1-qL, suggest that PSII is intact in *psbP2*, and that electron transport functions as effectively in the mutant as in the wild-type. PPL1 in *A. thaliana* (Ishihara *et al.* 2007) and PSBP1 in *C. reinhardtii* (de Vitry *et al.* 1989; Mayfield *et al.* 1987), were shown to alter PSII stability, a process which could alter singlet oxygen production in the cell. Results obtained from photoinhibition experiment presented in Figure 4.15 show only minor differences in the rate of PSII damage in the *psbP2* strain. Additionally, the rate of recovery from photoinhibition was due to the re-synthesis of PSII and was identical in the mutant and wild-type strains.

In order to further examine the functional complementation of the *psbP1* and *psbP2* genes, and to eliminate the possibility that they could be interchangeable parts of the photosynthetic machinery, the *psbP2* mutant was transformed with the pP1-Hyg3 plasmid (*PSBP1* gene in pHyg3). Transformants were subjected to experimental conditions that were shown previously to induce *GPX5-ARS2* expression in signalling reporter lines (as stated in Chapter 2, *Section 2.3.2*) or lines successfully but partially rescued with pP2-T7Arg7 or pP2-Hyg3 (see *Section 4.3.6*) followed by the arylsulfatase assay. However, introducing the wild-type *PSBP1* gene into *psbP2* did not recover abrogated $^1O_2$-inducible signalling. These results indicate that the mutation in *PSBP2* is responsible for the mutant phenotype and *PSBP1* cannot complement its function.

In a reciprocal experiment, the *CC-4142* (*oee2*) strain that carries a *psbP1* mutation, as identified previously by Mayfield *et al.* (1987) was transformed independently with *PSBP1* and *PSBP2* genes in the pHyg3 plasmid. While transformation of *CC-4142* with *PSBP1* was successful, recovering the wild-type growth phenotype in approximately 33% of the transformants examined, the transformation with *PSBP2* proved to not affect the increased light sensitivity in *oee2*. Thus, it can be assumed that the PSBP1 protein encoded by the gene carried in the pHyg3 plasmid was fully functional, supporting data obtained from analysis of the *psbP2* mutant line transformation with pP1-Hyg3.

Thus, results obtained from both experiments aimed to examine complementation of PSBP1 and PSBP2 confirmed that the function of a mutated PSBP1 cannot be complemented with PSBP2 and *vice versa*. Together with previous experiments,

comparing photosynthetic characteristics or growth responses to HL conditions, it is indicative of PSBP2 not being a part of the oxygen evolving complex of PSII. Therefore it can be concluded that these closely related proteins, PSBP1 and PSBP2, play distinct roles in *C. reinhardtii*. This set of experiments also acts to confirm that the partial rescue of the *psbP2* strain using the *PSBP2* gene is real and not due to spurious effects of the vector or transformation protocol.

Further evidence pointing towards the role of PSBP2 as a component of the singlet oxygen-dependent signalling system is that GPX5-ARS2 activity was not restored by treatment with photosensitizers which produce exogenous singlet oxygen. Thus, one would surmise that if the *psbP2* strain were not producing the expected *GPX5-ARS2* response due to an inherent lack of singlet oxygen production, treatment with rose bengal or neutral red, would induce the signal. While both photosensitizers were able to induce *GPX5* driven ARS activity in the *sigRep2* strain this was not the case in *psbP2*.

Interestingly, results obtained from an experiment aimed to determine if the *psbP2* strain was more sensitive to $^1O_2$ stress suggest that the PSBP2 protein is not absolutely required in the $^1O_2$-acclimation mechanism. Following the protocol of Ledford *et al.* (2007), an attempt was made to induce a singlet oxygen acclimation response in the *psbP2* strain. It was expected that the mutant would be less tolerant of additional $^1O_2$ because it would have little to no GPX5. However, it was not observed and there are possible reasons for this apparent discrepancy. On one hand, GPX5 may only play a cursory role in the overall singlet oxygen acclimation process. While this would seem unlikely due to its rapid and extensive up-regulation at the level of transcript accumulation, other systems may take over to protect the cell. Alternatively, it is possible that the accumulation of one form of GPX5 may occur at a normal level, and thus protect the cell from singlet oxygen-mediated damage. However, this possibility could not be verified because of lack of antibodies that would allow determining actual GPX5 protein level in the cell. Fischer *et al.* (2009) demonstrated that two forms of the *GPX5* transcript were produced when *C. reinhardtii* cells were challenged with $^1O_2$. As suggested previously (Dayer *et al.* 2008; Fischer *et al.* 2009), the *GPX5* gene may encode a dual-targeted protein (chloroplast and cytoplasm) via alternative expression of the two independent transcription (TSS-1 and TSS-2) and translation (ATG-1 and ATG-2) start

sites as depicted in Figure 4.20. The TSS-1 called Initiator (Smale 1997) is responsible for expression of the longer GPX5 transcript that contains the first translation start site (ATG-1) 147 bp upstream of the second translation start site (ATG-2; Figure 4.20). This longer sequence encodes an extra 49 amino acid N-terminus motif, with a putative cleavage site at position 48 of the protein, that is predicted to target the GPX5 protein to the chloroplast (Figure 4.20; Fischer *et al.* 2009). The TSS-2 is located 45 bp downstream of the TSS-1 and thus encodes a shorter version of the *GPX5* transcript that contains only the second translation start site (ATG-2). As shown by Fischer *et al.* (2009) both long and short versions of the *GPX5* transcript are expressed at the same level in the dark, under low-light conditions or when treated with *t*-BOOH. However, exposure to high-light or RB treatment induces an approximately 3-fold up-regulation in the case of the long transcript levels and a 12 to 13-fold increase in the case of the shorter transcript (Fischer *et al.* 2009). The authors proposed that the longer version might be targeted to the chloroplast, and the shorter version to the cytosol, where it could play a role as a TRX-dependent peroxidase (Fischer *et al.* 2009).

The *GPX5* 5' regulatory region used in study presented here was amplified with KW64 and KW65 primers and contains all the regulatory elements required for $^1O_2$-induction (Figure 4.20). Primers used in sqRT-PCR for amplification of the short (KW235 and KW236) and long (KW233 and KW234) versions of the endogenous *GPX5* 5' RR transcript, as well as transcript of the short (KW230 and KW237) and long (KW233 and KW237) version of the introduced *GPX5* 5' fused with *ARS2* gene are indicated in Figure 4.20. Thus, if the 70 bp fragment of the 147 bp encoding chloroplast transit peptide of the GPX5 included in the reporter gene is sufficient to direct the GPX5-ARS2 to the chloroplast in the absence of *psbP2*, rather than exported to the periplasmic space, it might not be possible to detect the protein using the arylsulfatase assay. In this case, enough GPX5 may be translated and imported into the chloroplast to effectively protect the cells from oxidative challenge following the acclimation process. Therefore the PSBP2 protein may not be a critical factor for the accumulation of chloroplast localized GPX5; however it still may be playing a critical role in regulating which forms of GPX5 are being produced under particular oxidative stress conditions.

An increased accumulation of the long version of the native *GPX5* transcript was observed, at roughly the same levels, in *CC-3395*, *sigRep2*, and *psbP2*, both from GL and exposure to RB treatment, while the lower levels were observed in *RΔsig-P2H-4* (Figure 4.21b). The lack of the higher expression levels of the long *GPX5* when exposed to RB is in agreement with the results obtained by Fischer *et al.* (2009). The short version of the native *GPX5* transcript displayed higher induction upon exposure to RB in *CC-3395* and *sigRep2* than in *psbP2* or *RΔsig-P2H-4*, which was expected based on the findings of Fischer *et al.* (2009), and it also confirms diminished or abrogated $^1O_2$-inducible signalling in *psbP2*. However, these results are inconclusive in case of the *RΔsig-P2H-4* line, which displayed at least partially rescued $^1O_2$-inducible signalling, observed by means of ARS2 activity (Figures 4.10D and 4.12), and thus levels of short and long endogenous *GPX5* transcripts were expected to be at the similar levels to these observed in the *CC-3395* or the *sigRep2* line.

Analyses of the expression with primers designed to amplify short (KW230) or long (KW233) versions of the *GPX5* fused with *ARS2* (KW237; Figure 4.20b and c) indicated increased accumulation of the short *GPX5-ARS2* transcript in the case of lines treated with RB, with clearly pronounced higher levels observed in *sigRep2* line when compared to *psbP2* (Figure 4.21c). These findings are consistent with observations from experiments examining *GPX5-ARS2* induction on plates or cell suspension cultures by $^1O_2$ generated by NR (Figures 2.3, 2.5, 4.8, 4.9, 4.10, and 4.12) or by RB (Figure 4.9), and also in agreement with what was demonstrated by Fischer *et al.* (2009). In case of the *RΔsig-P2H-4* line and treatment with RB, the short *GPX5-ARS2* transcript was observed to be at the lower level than in *sigRep2* but higher than in the *psbP2* mutant line. These findings are consistent with the results obtained from the rescue experiments examining the *GPX5-ARS2* inducibility by $^1O_2$ pronounced as measured ARS2 activity. Expression of the short *GPX5-ARS2* from the lines grown under GL was lower in *sigRep2* and *RΔsig-P2H*-4 when compared to the RB treatment, while not detectable in case of *psbP2* (and *CC-3395* used as a negative control). Analogically to the experiments examining endogenous short *GPX5* transcript levels, primers for the short version of the *GPX5-ARS2* amplify both short and long fragments.

Analyses of the long version of the *GPX5-ARS2* indicated presence of the transcript in case of *sigRep2*, *psbP2* (< *sigRep2*), and *RΔsig-P2H-4* (< *psbP2*) but with the similar trend in case of lines grown under GL conditions compared to RB treatment. Similarly to the results obtained from examination of the long version of the endogenous *GPX5*, and as was demonstrated by Fischer *et al.* (2009), the expression of the long version of the *GPX5-ARS2* transcript is not expected to be up-regulated by $^1O_2$.

In conclusion, the RESDA-PCR-based technique allowed identification of the mutation in the *PSBP2* gene encoding a protein involved in regulation of the *GPX5-ARS2* reporter gene and by extension *GPX5* expression. However, it does not abolish completely the transcript presence of the native *GPX5* or *PSBP2* as expected. Nevertheless, the decrease of the short endogenous *GPX5* and *GPX5-ARS2* mRNA in *psbP2* mutant line was observed in case of the RB treatment. Results presented here, obtained from sqRT-PCR experiments, examining expression of short and long versions of the *GPX5* or *GPX5-ARS2* showed the trend demonstrated in previous studies conducted using qRT-PCR (Fischer *et al.* 2009). However, the differences in expression patterns were not pronounced to such an extent as shown by Fischer *et al.* (2009), possibly because of the different experimental design and methodological approach.

CHAPTER 5.
GENERAL DISCUSSION

ROS generated in the chloroplast are known to induce changes in photosynthetic gene expression. Evidence suggests the involvement of $^1O_2$ and H_2O_2 in separate chloroplast-to-nucleus retrograde signalling pathways (Fischer *et al.* 2007b; Galvez-Valdivieso and Mullineaux 2010; Jaspers and Kangasjarvi 2010; Moller and Sweetlove 2010; Pfannschmidt 2010; Reinbothe *et al.* 2010). However, there is still little genetic evidence concerning mechanisms that govern the signalling pathways that communicate ROS information between the chloroplast and nucleus in any organism.

The research presented here focused on the role of $^1O_2$ in the chloroplast-to-nucleus retrograde signalling pathway in *C. reinhardtii*. In this organism, the *GPX5* gene was shown to be strongly up-regulated at the transcript level in response to $^1O_2$ (Leisinger *et al.* 2001). Moreover, it was demonstrated that the $^1O_2$-acclimation mechanisms exist triggering a response that results in increased tolerance to $^1O_2$ exposure (Ledford *et al.* 2007). However, many key questions remain. For example, what are the components involved in transmitting the signals and/or altering the expression of *GPX5*? How are the $^1O_2$ levels in the cell being detected? How is the response initiated? With the discovery presented here, describing identification of the PSBP2 protein as a component of $^1O_2$-induced retrograde signalling, it was possible to address the first of these questions. This represents an important first step in identifying the complex mechanisms responsible for the detection of changes in $^1O_2$ levels generated in the chloroplast. The evidence gathered so far points to several pathways or factors that may be involved but there is no indication that these mechanisms act separately.

In research presented here, a reporter gene was engineered, with the 5' regulatory region of the *GPX5* fused to a promoterless copy of the *ARS2*, and used to transform *C. reinhardtii* cells to generate stable and reproducible $^1O_2$-responsive reporter strains (Leisinger *et al.* 2001; described in Chapter 2). Genetically transformed lines were subjected to a series of experiments aimed to test and to verify inducibility of the introduced *GPX5-ARS2* reporter gene by $^1O_2$. Results obtained confirmed specificity of $^1O_2$-dependent induction of the *GPX5* regulatory region which was detectable by

chromogenic reaction of the product of ARS2 enzyme activity. Thus, these research steps led to generation of lines which allowed monitoring changes in nuclear-encoded *GPX5* expression in response to $^1O_2$.

A secondary insertional mutagenic screen, described in Chapter 3, led to the identification of a number of secondary mutants which failed to display *GPX5* driven ARS2 activity when challenged by NR and ML or exposed to ML (Figure 3.1). Some of these lines potentially carried a mutation that disrupted gene encoding a protein component involved in transmitting changes in $^1O_2$ concentration and thus abrogating the nuclear expression of *GPX5*.

Finally, PCR-based techniques allowed identification of the mutagenized gene in one of the mutant strains. The identified nuclear genome-localized gene encodes the PSBP2 protein which belongs to a protein family grouped together based on the sequence similarity of a domain from the PSBP1 (OEE2) protein. The PSBP1 protein is a nuclear encoded extrinsic subunit of PSII that facilitates formation of the oxygen evolving complex and was shown to optimize the water-splitting reaction *in vivo* (Ishihara *et al.* 2007), it may also be responsible for stabilization of the PSII (Ifuku *et al.* 2005). The PSBP2 is paralogous to the PSBP1, yet those two closely related proteins seem to play distinct roles in the cell as described in Chapter 4. In *A. thaliana*, there is a small family of proteins which consists of 10 members, all containing a PSBP motif (Ishihara *et al.* 2007). The gene family was divided into 3 subgroups; *PSBP1* and *PSBP2* encode versions of the 23 kDa protein, two other proteins, shown to function in the assembly of photosynthetic protein complexes, were placed in a category called PSBP-like or PPL, and lastly, a group of six PSBP-domain (PPD) proteins (Ishihara *et al.* 2007). However, there is no indication that either of these proteins is involved in signalling pathways in *A. thaliana* and nothing has been reported regarding the six genes placed in the PPD group. The duplication and evolution of *PSBP* genes appears to have first occurred prior to the split between algae and plants, as *C. reinhardtii* appears to express five genes encoding proteins containing PSBP domains in addition to its single *PSBP1* gene (Ifuku *et al.* 2008).

Figure 5.1 depicts a phylogenetic tree generated using the amino acid sequences of the PSBP-like proteins of *C. reinhardtii* (Cr) and *A. thaliana* (At). The CrPSBP2

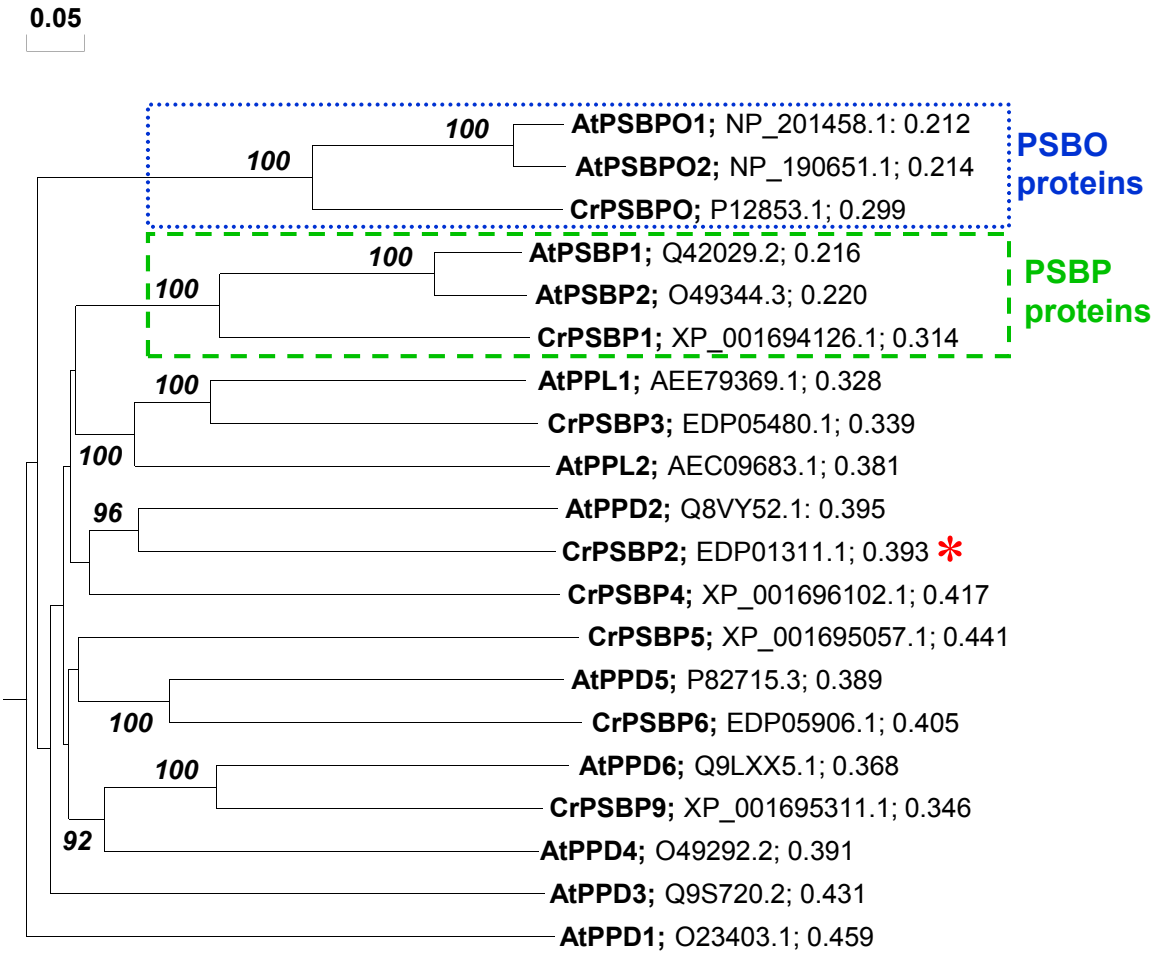

Figure 5.1. A phylogenetic tree constructed for comparative purposes, based on amino acid sequences of the PSBP protein families of *C. reinhardtii* and *A. thaliana*. Protein name, accession number (NCBI protein sequence database; http://www.ncbi.nlm.nih.gov/protein), and a sequence weight are indicated. The comparison was made using the DNAMAN program, Version 6 (Lynnon Corporation, Pointe-Claire, QC, Canada). Bootstrap values from a 1000 iteration analysis are shown in italics. The PSBP proteins that are components of the PSII oxygen evolving complex and required for optimal PSII function are indicated in a green box with dashed lines. The PSBO proteins that are also components of the oxygen evolving complex, but not part of a larger gene family were used as the out-group in the tree and are indicated by a blue box with dotted lines. *C. reinhardtii* PSBP2 protein identified in this study as required for $^1O_2$-induced chloroplast-nucleus retrograde signalling is marked by red asterisk. The alignment of sequences used to construct the phylogenetic tree is presented in Figure E10.1.

protein branches further away from the CrPSBP1 protein grouping more closely with CrPSBP6 than CrPSBP1. The CrPSBP2 protein appears to have highest identity to the *A. thaliana* PPD2 protein, the function of which still remains unknown.

Protein oxidation by ROS leads to specific modifications which depend on the given ROS reactivity (Davies 2004; Halliwell and Gutteridge 2007). Thus oxidized peptides would be good candidates for secondary messengers in ROS signalling pathways because they would not only carry information about the specific ROS but also encode information with regard to its source and concentration (Moller and Sweetlove 2010). There is still not sufficient evidence but it could be hypothesized that the nuclear encoded PSBP2 protein identified in this study might play a role of a very specific sensor of the changes in chloroplastic $^1O_2$ concentration. If indeed targeted to the chloroplast, as predicted by bioinformatics analyses, it may undergo $^1O_2$-specific modifications, and then interact with other signalling components, initiating signalling cascades outside of the plastid, leading to the induction of transcription factors that up-regulate *GPX5* transcript accumulation, and modulating the translational control mechanism that theoretically could exist to further control *GPX5* expression.

The research presented here identifying the involvement of PSBP2 in chloroplast-to-nucleus signalling induced by $^1O_2$ raised questions that need to be answered within the framework of the research presented in this thesis. One critical question concerns the applicability of using the *GPX5* regulatory region to drive *ARS2* expression because of its putative dual localization. It is not clear whether diverse localization can affect the detectability of ARS2 activity. This could be determined by development of antibodies that would allow one to determine the level of the protein present in the cell, juxtaposed with its transcript level, in response to changes in $^1O_2$ concentration. This could explain whether GPX5 regulation takes place at the transcriptional or translational level, or both. Cell fractionation and immunoblotting could then also be used to determine where in the cell the GPX5 protein is located. Alternatively, localization of the GPX5 could be examined by separate fusion of the putative chloroplast-targeting sequence and fragment of the *GPX5* gene encoding cytosolic version of the protein (Fischer *et al.* 2009) to a fluorescent tag.

Another important question concerns the exact role of the PSBP2 protein in detecting $^1O_2$ levels in the cell. One factor that needs to be determined is the actual subcellular localization of the PSBP2 protein. Bioinformatic analyses predict PSBP2 to be chloroplast localized, however experiments need to be performed to confirm this hypothesis. Again, the subcellular localization of the PSBP2 protein could be examined using fluorescent tag fusion constructs. Alternatively, because confocal imaging of *C. reinhardtii* chloroplast proteins can prove difficult, the development of antibodies against the PSBP2 protein or epitope tagging would allow one to determine its location by immunoblotting of subcellular fractions.

Access to antibodies raised against PSBP2 would also allow one to perform co-immunoprecipitation (Co-IP) experiments to identify proteins that interact with PSBP2. It is highly unlikely that the PSBP2 protein is the only component involved in the regulation of *GPX5* expression induced by $^1O_2$. In other words, it must act as part of a signalling pathway. The separation of interacting proteins by Co-IP and SDS-PAGE, followed by their identification by mass spectrometric analysis of the protein bands may prove to be effective way of discovering the exact role of PSBP2 and how the $^1O_2$-dependent signal leaves the chloroplast.

Finally, because the PSBP2 protein of *C. reinhardtii* appears to be most closely related to the PPD2 protein of *A. thaliana* (Figure 5.1), it will be interesting to examine whether the PPD2 protein plays a role in $^1O_2$-sensing or signalling in *A. thaliana*. A potential PPD2-knock out line can be obtained from the Riken Bioresource Center (BRC). Examination of the PPD2 function in higher plants would allow one to understand the evolutionary role of this protein in $^1O_2$-induced retrograde signalling.

In conclusion, obtained results suggest that the PSBP2 protein of *C. reinhardtii* is a novel component of $^1O_2$-dependent chloroplast-nucleus retrograde signalling. However, the exact role of this protein in conveying information with regard to $^1O_2$ produced by exposure to high-light or by exogenous photosensitizers, is not known at this time and will require extensive future work.

6. APPENDIX A. Supplementary Tables

Table A6.1 Tris-acetate-phosphate medium (TAP). The stock solutions (SL) of Beijerinck salts and trace elements were prepared at the given concentrations and added at the following volumes up to 1 L of final medium volume (Andersen 2005; Gorman and Levine 1965; Harris 1989; Hutner *et al.* 1950; Sueoka 1960)

Stock solution (SL)	Volume	Component	Concentration in SL	Conc. in final medium
Tris base	2.42 g	$H_2NC(CH_2OH)_3$ (Tris(hydroxymethyl)-aminomethan (BioShop))		20 mM
TAP-salts	25 mL	NH_4Cl (EMD Chemicals)	16 g/L	7.47 mM
(Beijerinck salts)		$MgSO_4 \cdot 7H_2O$ (J.T. Baker)	4 g/L	0.83 mM
		$CaCl_2 \cdot 2H_2O$ (EM Industries, Darmstadt, Germany)	2 g/L	0.45 mM
2.7 M Phosphate solution	1 mL	K_2HPO_4 (BioShop) 28.8 g in 100 mL	1.65 M	2.7 mM (total)
(pH 7)		KH_2PO_4 (J.T. Baker) 14.4 g in 100 mL	1.05 M	
Trace elements solution	1 mL	$Na_2EDTA \cdot 2H_2O$ (Fischer Scientific Company)	0.5 g/L	134 µM
(Hunter trace elements)		$ZnSO_4 \cdot 7H_2O$ (J.T. Baker)	0.22 g/L	136 µM
		H_3BO_3 (J.T. Baker)	0.114 g/L	184 µM
		$MnCl_2 \cdot 4H_2O$ (J.T. Baker)	0.05 g/L	40 µM
		$FeSO_4 \cdot 7H_2O$ (J.T. Baker)	0.05 g/L	32.9 µM
		$CoCl_2 \cdot 6H_2O$ (J.T. Baker)	0.016 g/L	12.3 µM
		$CuSO_4 \cdot 5H_2O$ (J.T. Baker)	0.016 g/L	10 µM
		$(NH_4)_6MoO_3$ (EMD Chemicals)	0.011 g/L	4.44 µM
Acetic acid-glacial, (titrated to pH 7.1-7.2)	~1 mL	CH3COOH (J.T. Baker)		

Table A6.2. Primer sequences. Restriction enzyme sites underlined.

Primer	Target	Primer sequence	Chapter	*Section*
KW64	*GPX5* prom 5'	F: 5'-CCCTACGGTACCGGTACATGTTTAGAACC-3'	2	*2.2.4*
KW65	*GPX5* prom 3'	R: 5'-ACCGCAGATATCTGCAATCGTCGCTGG-3'	2	*2.2.4*
KW8	Ble^R, RESDA-PCR	F: 5'- GCTGCTTGAGACAGCGACAG-3'	4	*4.2.1*
PB16 [1]	Ble^R, RESDA-PCR	F: 5'-TTGGCTGCGCTCCTTCTGGCATTTAAATC-3'	4	*4.2.1*
Deg-*TaqI* [2]	TaqI, RESDA-PCR	R: 5'-CCAGTGAGCAGAGTGACGIIIIINNSWGTCGAA-3'	4	*4.2.1*
Deg-*AluI* [2]	AluI, RESDA-PCR	R: 5'-CCAGTGAGCAGAGTGACGIIIIINNSWCAGCTT-3'	4	*4.2.1*
Deg-*SacII* [2]	SacII, RESDA-PCR	R: 5'-CCAGTGAGCAGAGTGACGIIIIINNSCCGCGGW-3'	4	*4.2.1*
Deg-*PstI* [2]	PstI, RESDA-PCR	R: 5'-CCAGTGAGCAGAGTGACGIIIIINNSCTGCAGW-3'	4	*4.2.1*
Q0 [2]	RESDA-PCR	R: 5'-CCAGTGAGCAGAGTGACG-3'	4	*4.2.1*
PB28	*PSBP2*, cDNA	F1: 5'-GTGCTGCAGTGCCTGAGCGCTTC-3'	4	*4.2.5*
PB29	*PSBP2*, cDNA	R1: 5'-GATCAGCAGGAGGCAGCGTGTCC-3'	4	*4.2.5*
KW212	*PSBP2*, cDNA	F2: 5'-CCGTGCCACCTCGGCAATGT-3'	4	*4.2.3*
KW215	*PSBP2*, cDNA	R2: 5'-CAGCAGGAGGCAGCGTGTCC-3'	4	*4.2.3*
PB18S-F[3]	*18S*, cDNA	F: 5'-GATGGCTACCACATCCAAGGAA-3'	4	*4.2.3; 4.2.11*
PB18S-R[3]	*18S*, cDNA	R: 5'-AAGCGCCCGGTATTGTTATTTATT-3'	4	*4.2.3; 4.2.11*
KW198	PSBP2, 5'RACE	R1: 5'-CTTTCTCGA GCCGGCTGCACGTCAAACGTGGC-3'	4	*4.2.3*
KW173	PSBP2, 5'RACE	R2: 5'-CCTGCCGTTCCGAGCTAGAC-3'	4	*4.2.3*

Continues on the next page

Primer	Target	Primer sequence	Chapter	*Section*
KW209	Cre16.g678850, genomic	F: 5'-CCATACCCTGACTAACGTACTAGCATC-3'	4	*4.2.4*
KW210	Cre16.g678850, genomic	R: 5'-CTGTCGGACAAGTCGCTGGAGG-3'	4	*4.2.4*
KW207	Cre16.g678900, genomic and cDNA	F: 5'-GACGGTTGGGCGGCAACAAAGGG-3'	4	*4.2.4*
KW208	Cre16.g678900, genomic and cDNA	R: 5'-GACATCGCCGCCACGTCAAACG-3'	4	*4.2.4*
KW50	*CBLP*, cDNA	F: 5'-GACGTCATCCACTGCCTGTG-3'	4	*4.2.4*
KW51	*CBLP*, cDNA	R: 5'-CGACGCATCCTCAACACACC-3'	4	*4.2.4*
PB33	*PSBP2*, genomic	F1: 5'-CAGGGGCTGTGGAGGCCGC-3'	4	*4.2.5*
PB34	*PSBP2*, genomic	R1: 5'-ACAACGCCAGACATGTTGGGCTGCTC-3'	4	*4.2.5*
PB35	*PSBP2*, genomic	F2: 5'-GCGGGGCTGAAGAGTGCCATGAG-3'	4	*4.2.5*
PB36	*PSBP2*, genomic	R2: 5'-GCTCGTTGTGCCCAGGTGAGCATCC-3'	4	*4.2.5*
PB41	*PSBP1*, genomic	F: 5'- TTTTGATATCCTGTGGCTGCAGCGCTCTAGG-3'	4	*4.2.9*
PB42	*PSBP1*, genomic	R: 5'- TTTTGATATCAACGATGACGCCATGCCAGCC -3'	4	*4.2.9*
KW235 [3]	*GPX5*, short version	F: 5'-GCGGTCGCCAATAACCAAT-3'	4	*4.2.11*
KW236 [3]	*GPX5*, short version	R: 5'-AAGGGCTGTCCCGAAAGC-3'	4	*4.2.11*
KW233 [3]	*GPX5*, long version	F: 5'-AACCCTTTCACTCACATGCTGTCT-3'	4	*4.2.11*
KW234 [3]	*GPX5*, long version	R: 5'-CGAGCGGCGACAGGAGTA-3'	4	*4.2.11*
KW230	*GPX5*, short version	F: 5'-CTACTGAACCAGCGACGATTGC-3'	4	*4.2.11*
KW237	*ARS2*	R: 5'-GATGTACTTGTGCAGGCTGG-3'	4	*4.2.11*

[1] Dent *et al.* 2005; [2] Gonzalez-Ballester *et al.* 2005; [3] Fischer *et al.* 2009

7. APPENDIX B. Supplementary data for Chapter 2.

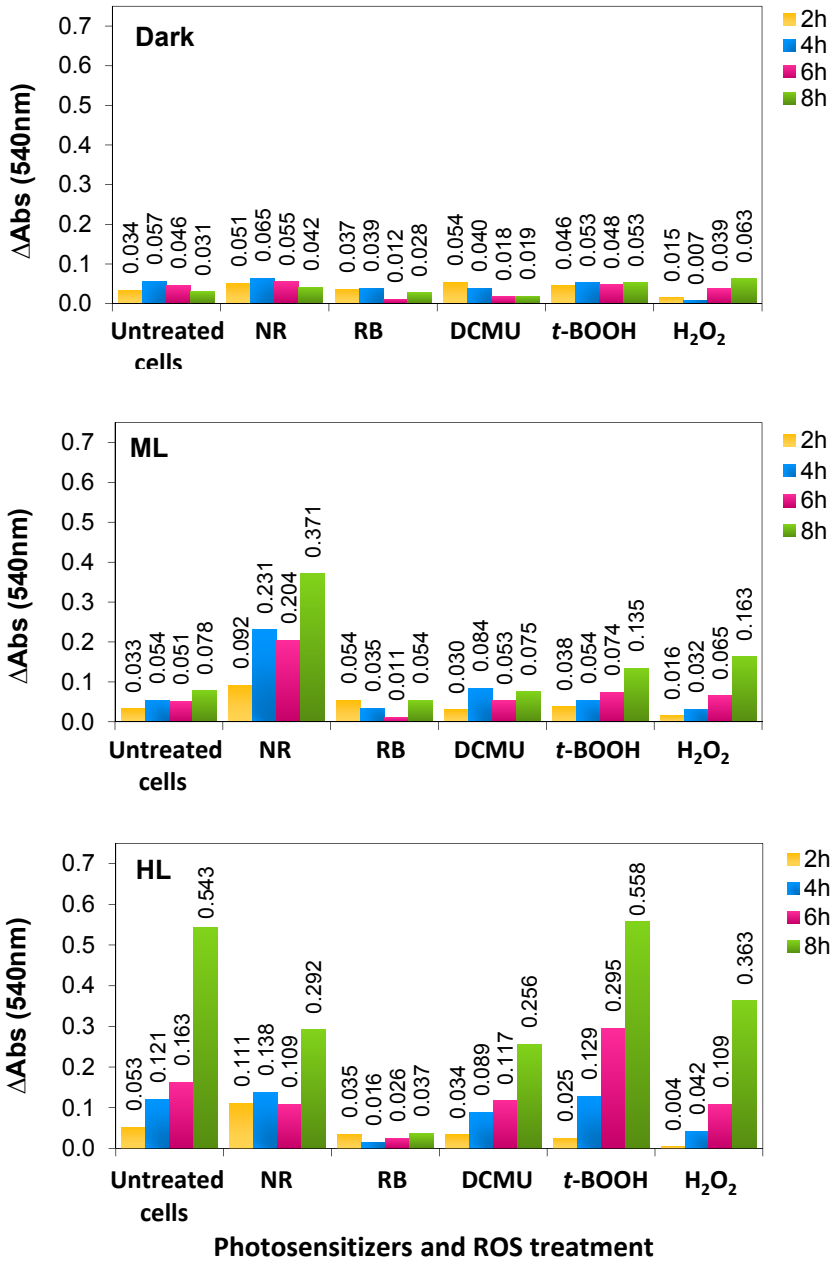

Figure B7.1 The first set of data for calculated means presented in Figure 2.5. Results obtained from the experiment examining the ARS2 activity in cell suspension cultures of the *sigRep2* line, exposed to chemical treatment combined with various light conditions: dark, ML (100 µmol photons m^{-2} s^{-1}) or HL (920 µmol photons m^{-2} s^{-1}). Aliquot of cells without the chemicals added was used as the control. Samples for absorbance measurements were taken every 2 hours.

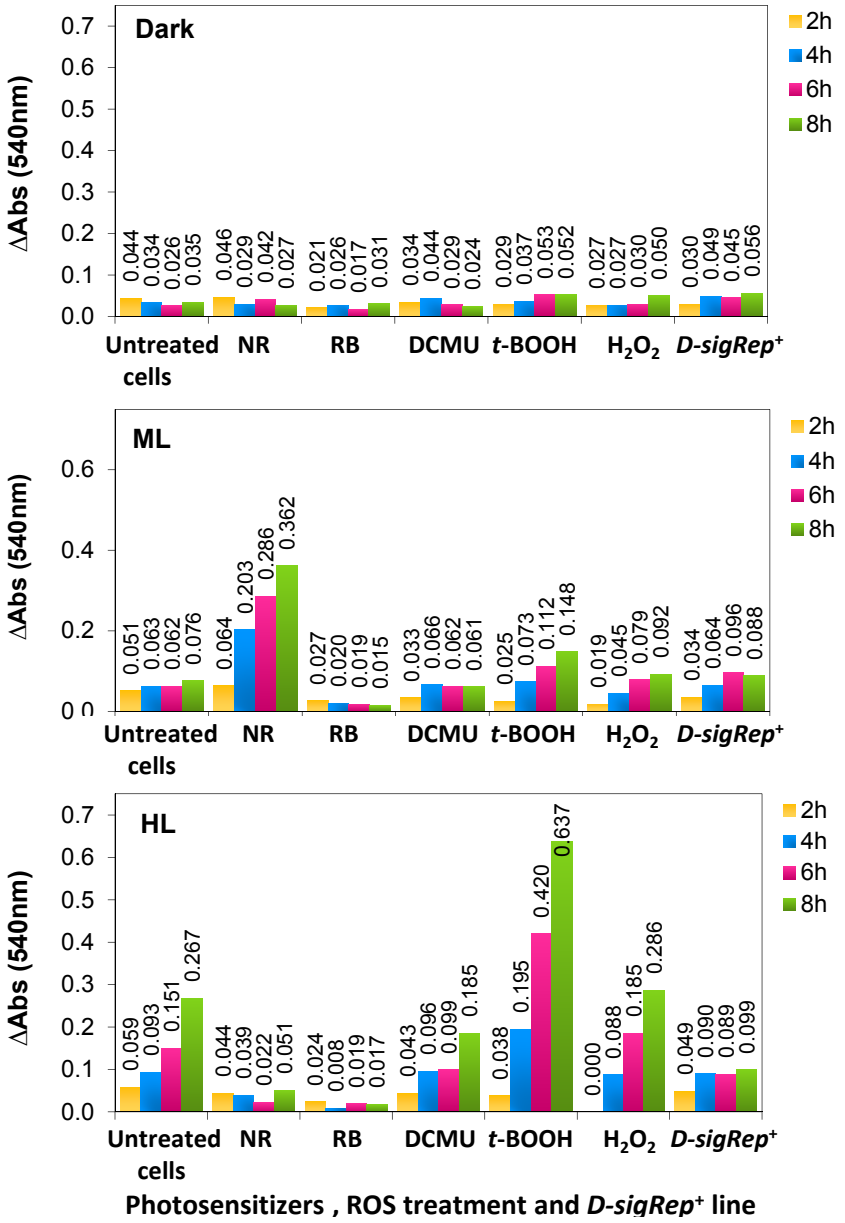

Figure B7.2 The second set of data for calculated means presented in Figure 2.5. Results obtained from the experiment examining the ARS2 activity in cell suspension cultures of the *sigRep2* line, exposed to chemical treatment combined with various light conditions: dark, ML (100 µmol photons m^{-2} s^{-1}) or HL (920 µmol photons m^{-2} s^{-1}). Aliquot of cells without the chemicals added was used as the control. The *D-sigRep*$^{+}$ expresses the *ARS2* gene under the control of the *PSAD* promoter and was used as a positive control, but these results are not included in calculated means presented in Figure 2.5. Samples for absorbance measurements were taken every 2 hours.

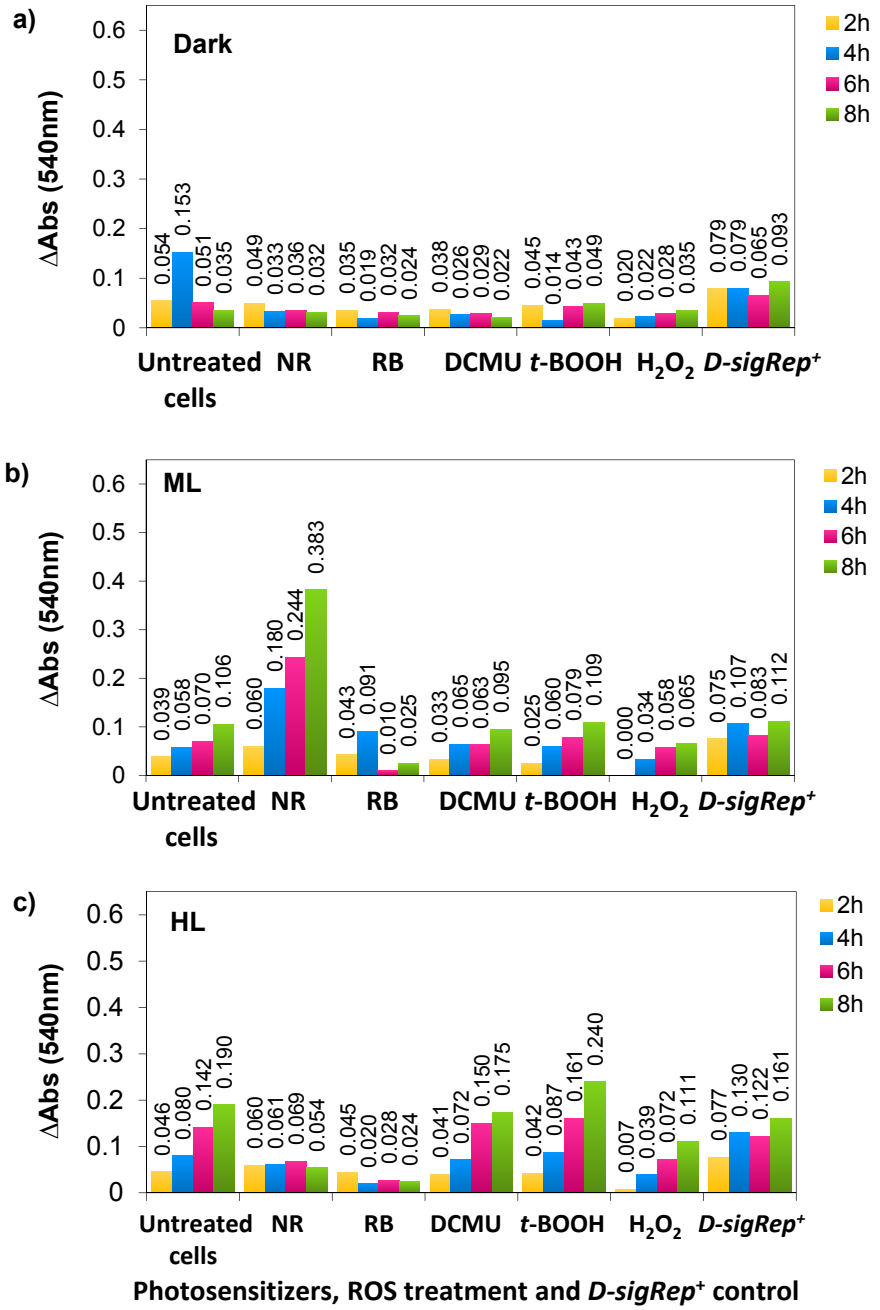

Figure B7.3 Data obtained from the experiment examining the ARS2 activity in the *sigRep13* line, determined in liquid cell cultures exposed to oxidative stress. After addition of NR, RB, DCMU, *t*-BOOH or H_2O_2 samples were incubated at 23°C in the **a)** dark, **b)** under ML (100 µmol photons m^{-2} s^{-1}) or **c)** HL conditions (920 µmol photons m^{-2} s^{-1}). Aliquot of cells without the chemicals added was used as the control. The *D-sigRep*$^+$ expresses the *ARS2* gene under the control of the *PSAD* promoter and was used as the positive control. Samples were taken every 2 h. The experiment was performed once.

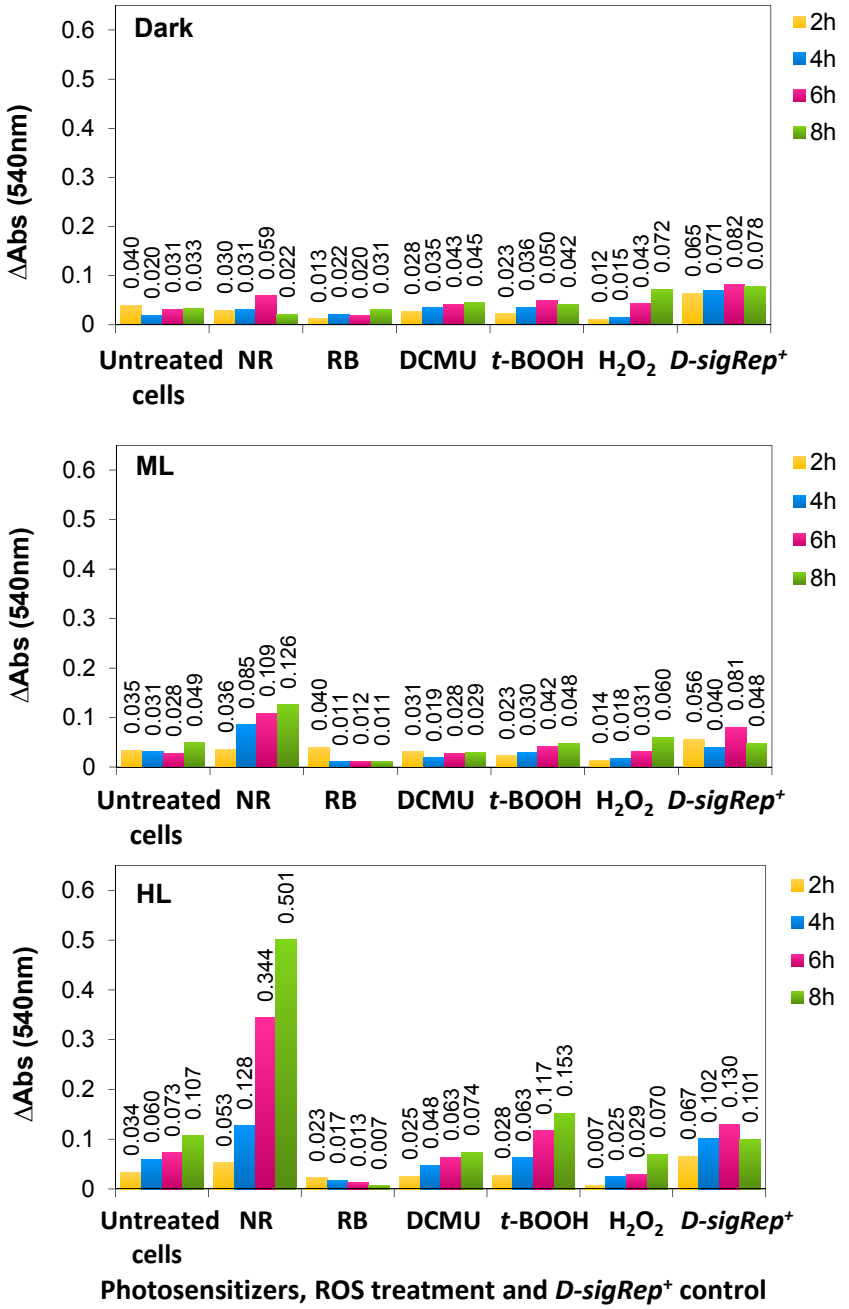

Figure B7.4 Data obtained from the experiment examining the ARS2 activity in the *sigRep18* line, determined in liquid cell cultures exposed to oxidative stress. After addition of NR, RB, DCMU, *t*-BOOH or H_2O_2 samples were incubated at 23°C in the **a)** dark, **b)** under ML (100 µmol photons m^{-2} s^{-1}) or **c)** HL conditions (920 µmol photons m^{-2} s^{-1}). Aliquot of cells without the chemicals added was used as the control. The *D-sigRep*$^+$ expresses the *ARS2* gene under the control of the *PSAD* promoter and was used as the positive control. Samples were taken every 2 h. The experiment was performed once.

8. APPENDIX C. Supplementary data for Chapter 3.

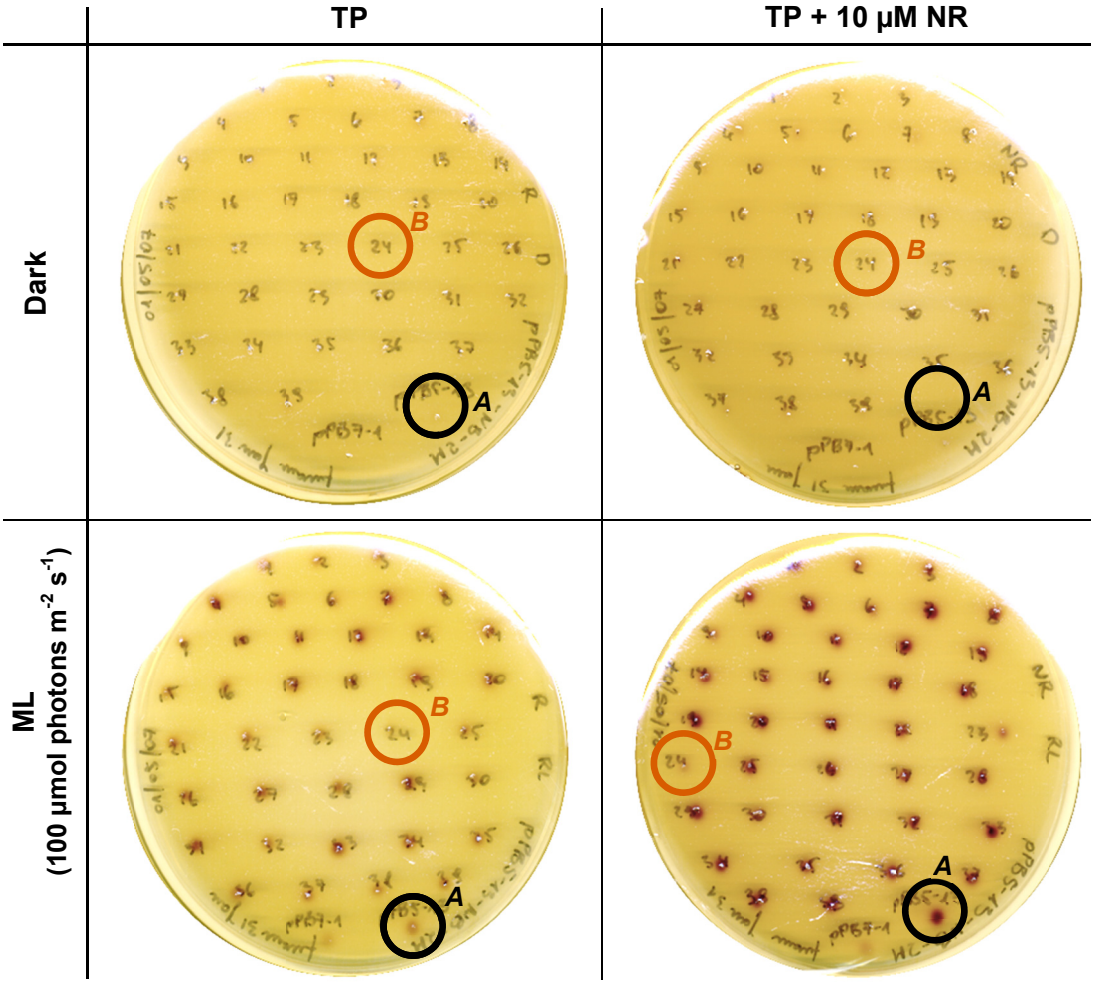

Figure C8.1 Selected *ΔsigRep13-24* (***B***) line with altered *ARS2* expression obtained from *sigRep13* subjected to secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep13* (***A***), showing $^{1}O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

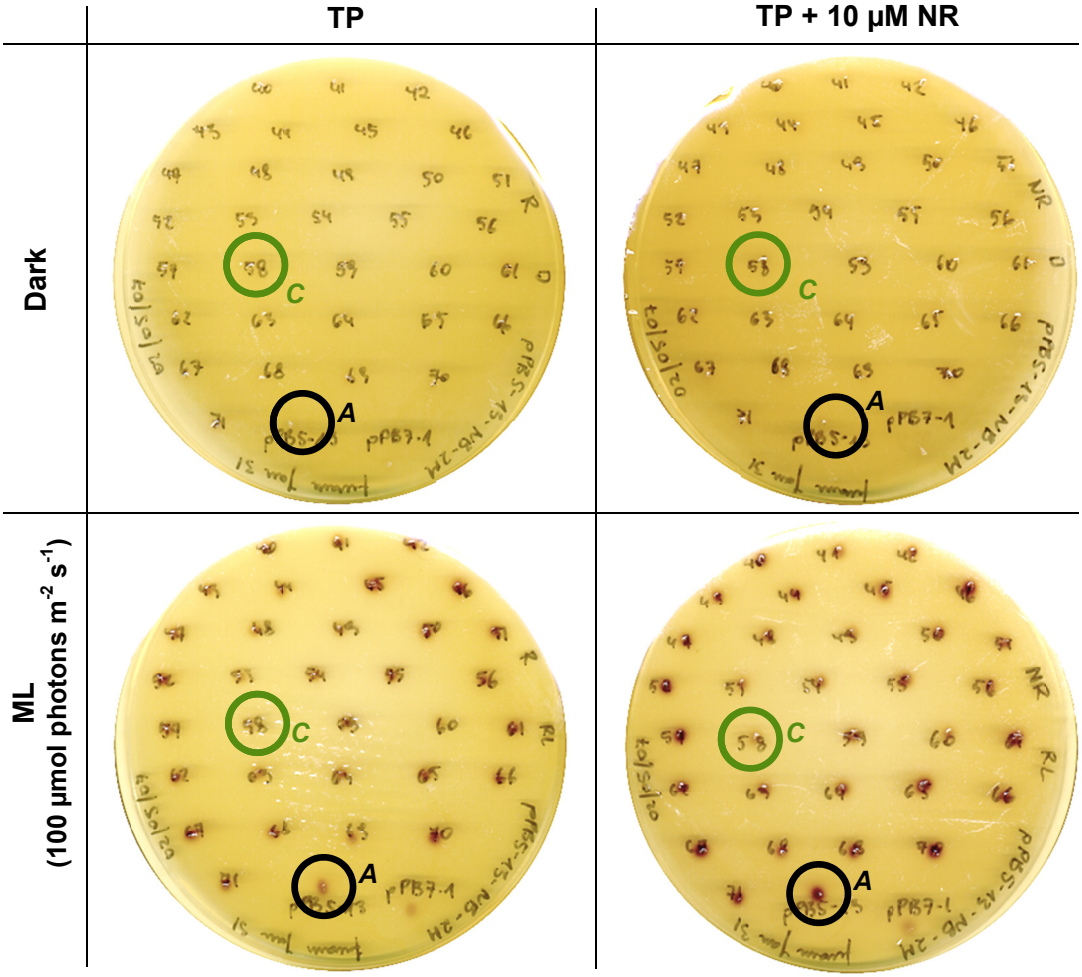

Figure C8.2 Selected *ΔsigRep13-58* (***C***) line with altered *ARS2* expression obtained from *sigRep13* subjected to secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep13* (***A***), showing $^{1}O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

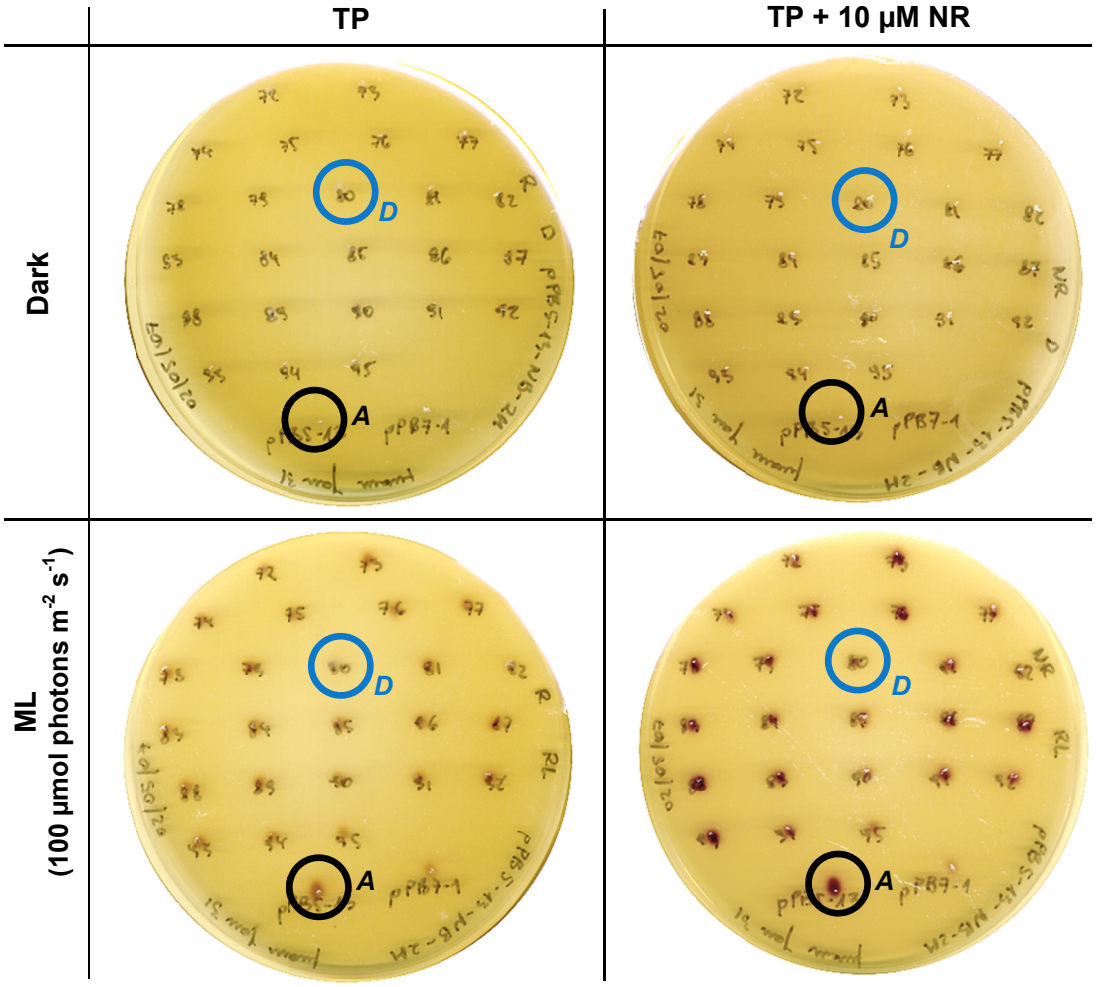

Figure C8.3 Selected *ΔsigRep13-80* (***D***) line with altered *ARS2* expression obtained from *sigRep13* subjected to secondary mutagenesis with Ble^R (Stevens *et al.* 1996). Line *sigRep13* (***A***), showing $^1O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

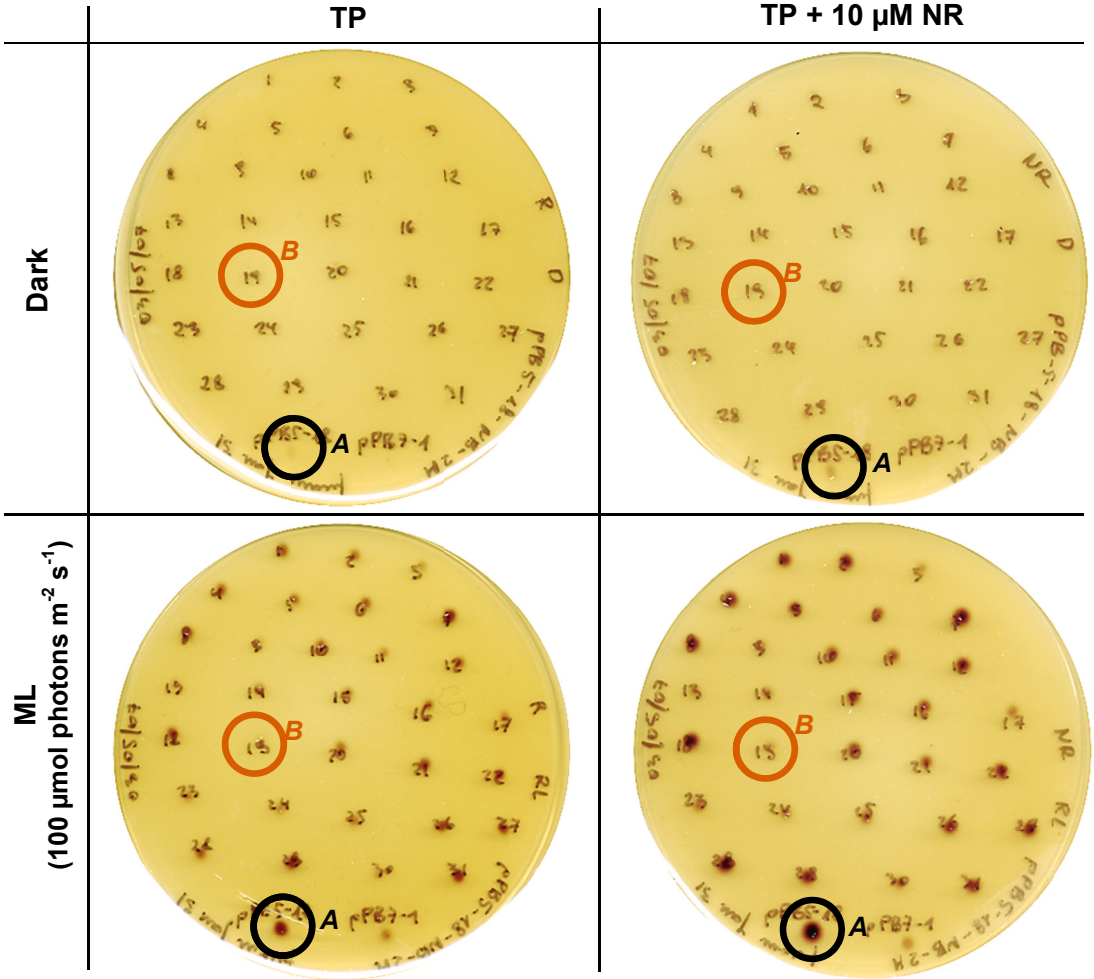

Figure C8.4 Selected *ΔsigRep18-19* (***B***) line with altered *ARS2* expression obtained from *sigRep18* subjected to secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep18* (***A***), showing $^{1}O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

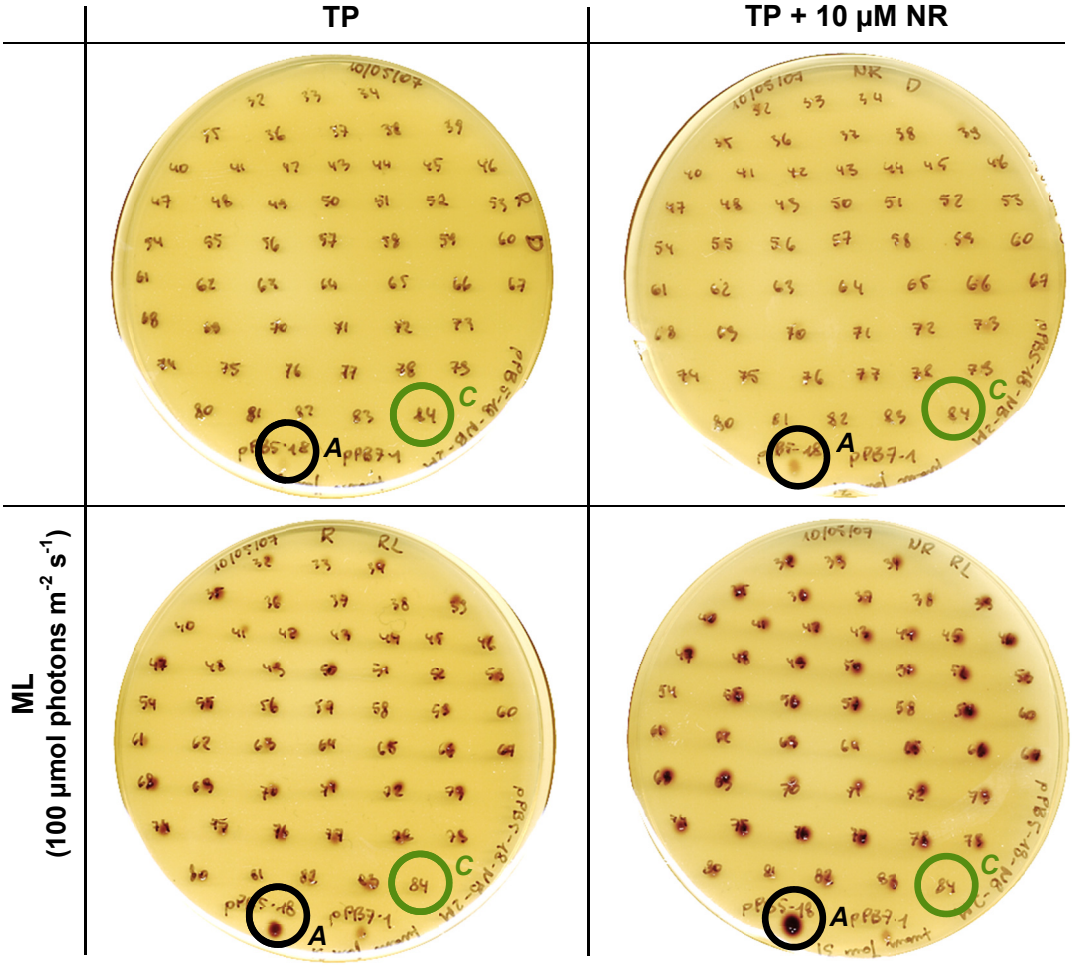

Figure C8.5 Selected *ΔsigRep18-84* (***C***) line with altered *ARS2* expression obtained from *sigRep18* subjected to secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep18* (***A***), showing $^{1}O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

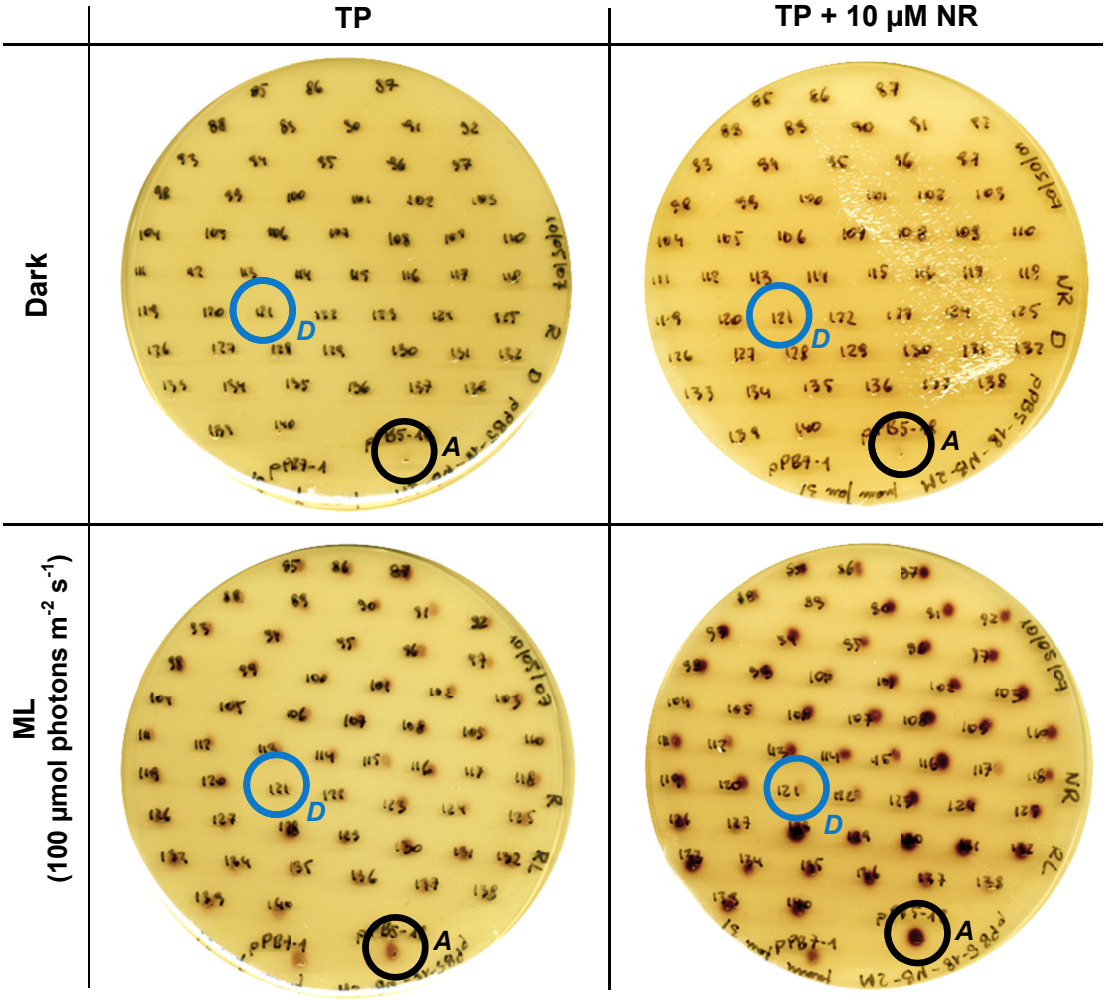

Figure C8.6 Selected *ΔsigRep18-121* (***D***) line with altered *ARS2* expression obtained from *sigRep18* subjected to secondary mutagenesis with Ble^{R} (Stevens *et al.* 1996). Line *sigRep18* (***A***), showing $^{1}O_2$-inducible *ARS2* expression, was used as a positive control. Examination of *GPX5-ARS2* expression was conducted as described in *Section 3.2.2*.

9. APPENDIX D. Supplementary data for Chapter 4.

```
   1    GTTCAAGCTT GCATGCACTG GCCGCCACGC GCTCCGGCTA CGCAGCCCGC
                        KW 212 →
  51    AGGCTTCCGC CGTGCCACCT CGGCAATGTG CGGAGCAGCA GCACCGACGC
 101    ATAAACGTGC TGCAGTGCCT GAGCGCTTCA ACAGACCCAA CTTCACTTGA
 151    GCAGCTTGCG GCGCGGCCAG CCCCAGAACA TGACGCCGCG GCCGCGCAAA
 201    TGCAGCGCGT GGCTGCAGCG GTGCCGCTCT CACGACGCAC CCTGCTCGCT
 251    CTCGCGTCCA TGGCCGTCGC CGTGGCCGCG CAGCAATTGG GCCCTGCCAG
 301    CACCGCTCGT GCAGAAGAGG CTTCTGCTGT GGCCGCCAGC ACCAGCGGCG
 351    CCGAGCAGGC GGCGGCCCCG GGGCCGTTGA CGCAGTACAA CAACACCGCC
 401    CAGAAGTACC AACTGCTTGT GCCGGCCAGC TGGGAGTCCA AGGGGAAAGC
 451    GGGCGCCGAC GCGCTGTTTG AGGACCCTGC GCGGCGCTCC ACGAGTGTGG
 501    GCGTGACGGT GAACCCGGTC AAGGTGGCGT CCATCGACAA GTTCGGGTCG
 551    CTGGCGGAGG TGGGCGACAA GCTGCTGGAG GCGGAGAAGA AGAAGGAGAG
 601    CACGCTGGCC GTGTCGCTGG TGTCTAGCTC GGAACGGCAG GGCGGCGCCG
 651    GTGCCAAGCT GTACGAGTAC GAGTACGAGC TGGACAGCAC CCGCGGCCGC
 701    AAGCGCATCC TCAACACAGT GACCATATTC AACTCCAGGC TATACATCCT
 651    AAACGCGGCA TACAAGTGCG AGAAGGAGGC GTGCGGCGAG GAGTCCCTGG
 801    CGGGTGTGCA GCTGCTGCGG CGCGTGGCGG CCACGTTTGA CGTGCAGCCG
 851    TAGAAGAGCC AACAGGAGGT CTGGAGCCCT AGATGCCTAG ATGTCATATT
 901    GATGGCGGAT GTGGCTTTGG CTGAGAAAGG GAAGCTGTAC GGAGGTACAT
 951    GCGGGAGGGC GAGGACTGGA GCTGTCTGGG GGGCAGAGCT GGAGGGGTGG
1001    TACGGCGCTG GGACACGCTG CCTCCTGCTG ATCCCGCGCC CCTGGGCAGT
                        ← KW 215
1051    CAGGCTGCGT GGGTGCTGGC GCATGGTTGG CATGGGGTCT TGGAGTGAAC
1101    GTCCATCTAT TGATACATGT AGCTCTCTCG GGCAGAGTGA CGCGGAGTTT
1151    GGTCCACCCC TCAGTTCACC ACTTCACCCA TATCCGTGCA TGGCGAGCAT
1201    GGTGATCGTA CGTGGCATGG GGCTGTCAGG CCACGAACAA GCAAGCATTC
1251    GCTACAGTTT AATTTATTAC ATATGTAAAG TGACGAAGAC
```

Figure D9.1 *PSBP2* mRNA sequence (1290 bp; Cre16.g678800; www.phytozome.net). Pairs of primers used for sqRT-PCR analysis of the PSBP2 transcript (*Section 4.3.2*) are indicated: KW212/KW215 in a grey-highlight and bold letters. Predicted ATG start codon is indicated in bold letters, underlined.

Table D9.1 Statistical analyses. *N* – sample number, *SE* mean – standard error of the sample mean, *StDev* – standard deviation, *DF* – degree of freedom, *SS* – sums square, *MS* – means square, *F* – F-value (Fisher test), *P* – probability (significance test).

Dark conditions

Results for no chemical treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	0.03489	0.00360	0.01080
	psbP2	9	0	0.00989	0.00320	0.00961
	RΔsig-P2H-4	9	0	0.02889	0.00531	0.01592
	RΔsig-P2H-17	9	0	0.02333	0.00508	0.01525
	RΔsig-P2H-20	9	0	0.02589	0.00169	0.00506

Results for NR treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	0.03711	0.00366	0.01099
	psbP2	9	0	0.01444	0.00294	0.00883
	RΔsig-P2H-4	9	0	0.02867	0.00682	0.02045
	RΔsig-P2H-17	9	0	0.02078	0.00695	0.02086
	RΔsig-P2H-20	9	0	0.01133	0.00307	0.00922

Two-way ANOVA: Absorbance versus chemical treatment, lines					
Source	***DF***	***SS***	***MS***	***F***	***P***
Chemical treatment	1	0.0001003	0.0001003	0.54	0.465
Lines	4	0.0060936	0.0015234	8.20	0.000
Interaction	4	0.0009983	0.0002496	1.34	0.261
Error	80	0.0148622	0.0001858		
Total	89	0.0220545			
S = 0.01363 R-Sq = 32.61% R-Sq(adj) = 25.03%					

		Individual 95% CIs For Mean Based on Pooled *StDev*
Chemical treatment	**Mean**	---------+---------+---------+---------+
No chemical treatment	0.0245778	(----------*----------)
NR treatment	0.0224667	(----------*----------)
		---------+---------+---------+---------+
		0.0210 0.0240 0.0270 0.0300

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		Individual 95% CIs For Mean Based on Pooled *StDev*
Lines	**Mean**	----+---------+---------+---------+-----
sigRep2	0.0360000	(-----*-----)
psbP2	0.0121667	(-----*------)
RΔsig-P2H-4	0.0287778	(------*-----)
RΔsig-P2H-17	0.0220556	(-----*-----)
RΔsig-P2H-20	0.0186111	(------*-----)
		----+---------+---------+---------+-----
		0.010 0.020 0.030 0.040

Moderate-light (ML)

Results for no chemical treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	0.06044	0.00760	0.02279
	psbP2	9	0	0.01311	0.00582	0.01745
	RΔsig-P2H-4	9	0	0.04578	0.00611	0.01832
	RΔsig-P2H-17	9	0	0.03922	0.00839	0.02516
	RΔsig-P2H-20	9	0	0.03456	0.00256	0.00767

Results for NR treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	0.5811	0.0341	0.1024
	psbP2	9	0	0.03511	0.00391	0.01173
	RΔsig-P2H-4	9	0	0.1439	0.0181	0.0543
	RΔsig-P2H-17	9	0	0.1854	0.0474	0.1422
	RΔsig-P2H-20	9	0	0.08989	0.00885	0.02655

Two-way ANOVA: Absorbance versus chemical treatment, lines					
Source	***DF***	***SS***	***MS***	***F***	***P***
Chemical treatment	1	0.63857	0.638573	175.80	0.000
Lines	4	0.96293	0.240732	66.27	0.000
Interaction	4	0.73684	0.184209	50.71	0.000
Error	80	0.29059	0.003632		
Total	89	2.62893			
S = 0.06027 R-Sq = 88.95% R-Sq(adj) = 87.70%					

		Individual 95% CIs For Mean Based on Pooled *StDev*
Chemical treatment	**Mean**	-------+---------+---------+---------+--
No chemical treatment	0.038622	(--*--)
NR treatment	0.207089	(--*-)
		-------+---------+---------+---------+--
		0.060 0.120 0.180 0.240

Continues on the next page

		Individual 95% CIs For Mean Based on Pooled *StDev*
Lines	**Mean**	+---------+---------+---------+---------
sigRep2	0.320778	(--*--)
psbP2	0.024111	(-*--)
RΔsig-P2H-4	0.094833	(-*--)
RΔsig-P2H-17	0.112333	(--*--)
RΔsig-P2H-20	0.062222	(--*--)
		+---------+---------+---------+---------
		0.00 0.10 0.20 0.30

High-light (HL)

Results for no chemical treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	0.2963	0.0306	0.0919
	psbP2	9	0	0.0707	0.0261	0.0784
	RΔsig-P2H-4	9	0	0.1627	0.0150	0.0449
	RΔsig-P2H-17	9	0	0.1421	0.0181	0.0543
	RΔsig-P2H-20	9	0	0.1110	0.0212	0.0635

Results for NR treatment:						
Variable	**Lines**	***N***	***N****	***Mean***	***SE Mean***	***StDev***
Absorbance	*sigRep2*	9	0	-0.0248	0.0208	0.0623
	psbP2	9	0	0.00278	0.00357	0.01072
	RΔsig-P2H-4	9	0	0.00844	0.00176	0.00527
	RΔsig-P2H-17	9	0	0.01300	0.00784	0.02353
	RΔsig-P2H-20	9	0	-0.00344	0.00426	0.01278

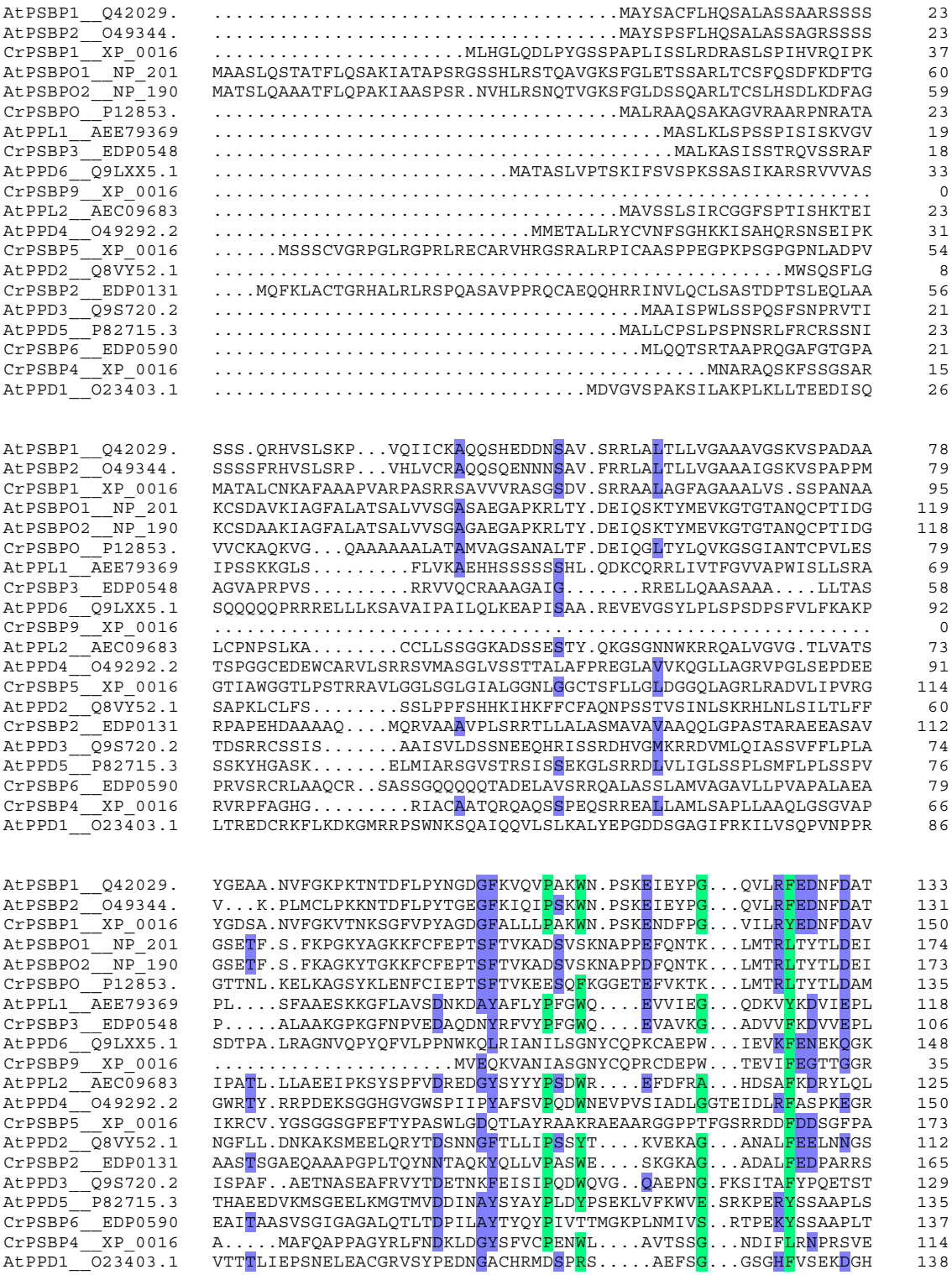
Two-way ANOVA: Absorbance versus chemical treatment, lines					
Source	***DF***	***SS***	***MS***	***F***	***P***
Chemical treatment	1	0.55712	0.557117	196.77	0.000
Lines	4	0.10235	0.025587	9.04	0.000
Interaction	4	0.16861	0.042153	14.89	0.000
Error	80	0.22650	0.002831		
Total	89	1.05458			
S = 0.05321 R-Sq = 78.52% R-Sq(adj) = 76.11%					

		Individual 95% CIs For Mean Based on Pooled *StDev*
Chemical treatment	**Mean**	---+---------+---------+---------+------
No chemical treatment	0.156556	(--*--)
NR treatment	-0.000800	(--*--)
		---+---------+---------+---------+------
		0.000 0.050 0.100 0.150

Continues on the next page

		Individual 95% CIs For Mean Based on Pooled *StDev*
Lines	**Mean**	-------+---------+---------+---------+--
sigRep2	0.135778	(-----*-----)
psbP2	0.036722	(-----*-----)
RΔsig-P2H-4	0.085556	(-----*------)
RΔsig-P2H-17	0.077556	(-----*------)
RΔsig-P2H-20	0.053778	(-----*------)
		-------+---------+---------+---------+--
		0.040 0.080 0.120 0.160

10. APPENDIX E. Supplementary data for Chapter 5.



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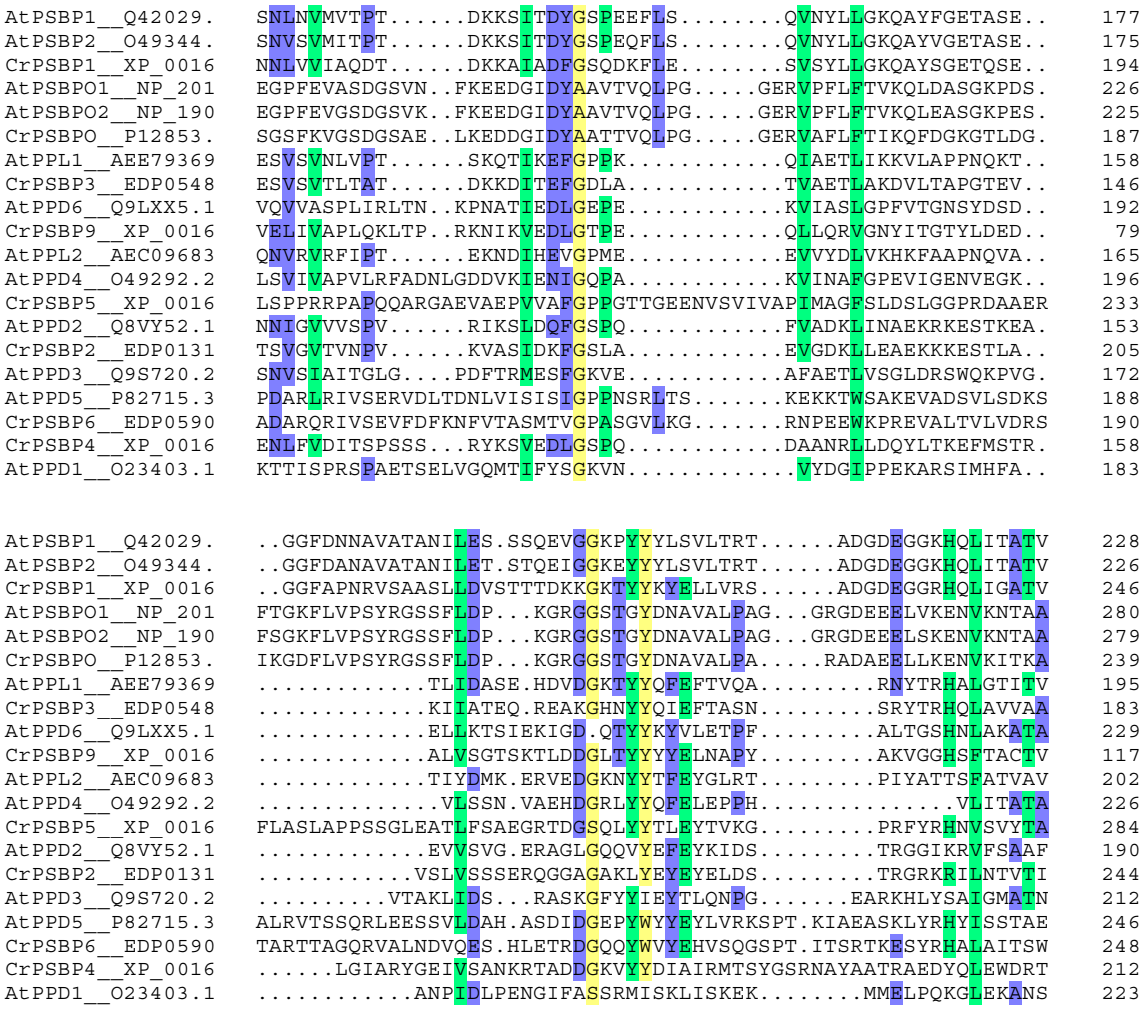

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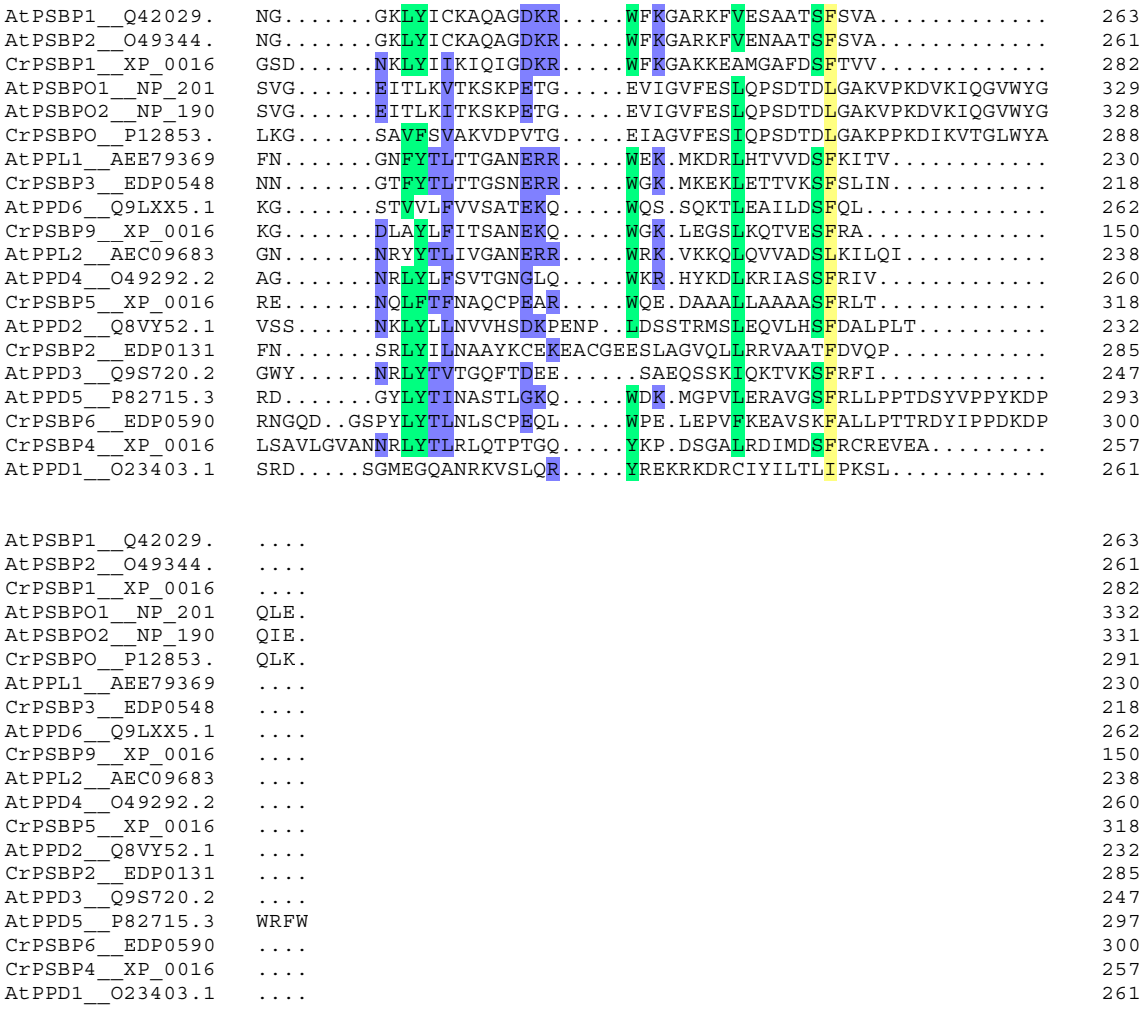

Figure E10.1 Aligned amino acid sequences of the PSBP protein family of *C. reinhardtii* and *A. thaliana* used to construct phylogenetic tree presented in Chapter 5 (Figure 5.1). The alignment was performed using DNAMAN program Version 6, based on sequences obtained from NCBI protein sequence database (http://www.ncbi.nlm.nih.gov/protein; protein names and accession numbers are indicated). The homology level ≥ 33% is indicated as a blue highlight, ≥ 50% as a green highlight, and ≥ 75% as a yellow highlight.

11. REFERENCES

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